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To the Readers of Acta Haematologica

Acta Haematologica has lost one of its editors-in-chief. The journal, during the past 10 years, mirrored the personality of Prof. Lüdin, whose qualities and achievements have been outlined by S. Moeschlin in the last issue of this journal.

When the publisher asked me to take over the task of the editorship, I hesitated. A number of questions arose which had to be answered first: Is it really justified to continue publication of *Acta Haematologica*? What is the place of this journal among the increasing number of haematological publications? What are its special characteristics? How can it fulfill the expectations of the readers? What should be the future policy of publication?

There are many possible answers to these questions. Let us consider few of them. *Acta Haematologica* was founded in 1948 as an international journal of haematology. At the time it was one of the first specialized journals connecting a subspeciality of internal medicine with experimental medicine ('Blood' had come out 2 years earlier. *Folia Haematologica* had disappeared during the war). Since then an overwhelming series of new periodicals has emerged. A few of them cover the whole field of haematology with particular emphasis on the clinical side. However most of these have primarily national character although they are, according to their broad international readership, open to authors from other countries and other parts of the world. On the other hand many new journals are devoted to particular aspects of haematology (immunology, haemostaseology, cytochemistry, etc.).

Therefore, we think that *Acta Haematologica* should keep its position as an international journal of general character. Many of us are convinced that in spite of continuing 'subspecialization' in our field, there will always be place for general topic 'Haematology'. *Acta Haematologica* should remain link between these subspecialities as well as between clinical and experimental haematology, opening its columns to all those who wish to present new data of general interest to an international audience. Papers dealing with clinical problems will be particularly welcome. However, *Acta Haematologica* will continue to publish experimental work as much as possible. The editors would also like to stimulate the publication of papers on selected chapters from the history of haematology or discussions on problems of edu-

cation and teaching in haematology However at the moment, it is not planned to change the character of *Acta Haematologica* fundamentally

Articles may be published under the following headings:

- original articles;
- brief preliminary communications
- illustrative case reports;
- letters or discussions on material published in *Acta Haematologica*.

In 1978, the journal will enter its 30th year This may give the opportunity to think over what the future trends of the journal well be, and to look out for new possibilities. Since it is our main concern to meet the expectations and wishes of our readers and of all those who potentially will join them, we are grateful for criticism and suggestions concerning the editorship of the journal.

The quality of a journal is defined by the standard of the published articles. It will be a challenge for the editors to keep or even to improve the standard of *Acta Haematologica*. This means that many papers will have to be rejected even if they are qualified as good. The editors will take freedom to base their decisions on the opinion of one or two referees.

It is evident that the whole burden of editorship cannot be worn by one or two persons alone. Therefore, I am very happy that a number of colleagues agreed to cooperate in this new field The editorial board will be further expanded in the forthcoming months. At this very moment the contributions of the members of the passed editorial board must be appreciated and, together with the publishers, I would like to thank them for their most valuable help

There are a few technical points to be added. From the beginning *Acta Haematologica* has been a multilingual journal. Whereas in the early years German was predominant, a continuous movement towards English took place, corresponding to the general trend in scientific communication. In agreement with the publisher we decided that articles in German or French will also be accepted in future, although English should be the main language of the journal. Every paper will include an informative summary in English. As to the linguistic details, we would like to leave it to the authors if they prefer the English or the American way of spelling.

Concerning units and measurements, we will follow the recommendations of the International Society of Haematology and adapt to the current practice of the main haematological periodicals.

One of the main difficulties for each scientific journal is the delay in publication. The editorial board together with the publisher will take every effort to shorten these delays. But authors should be reminded that it takes approximately 6 months from acceptance (not to be confused with receipt) of a paper until its publication. To clarify the situation the dates of receipt and final acceptance will be mentioned in future.

U. Bucher, *Berne*

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In vitro Labelling of Platelets

Experimental Study on Splenectomized Patients with Lymphomas Using Two Different Incubation Media

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Key Words. [51]Cr Lymphomas Platelet labelling Platelet production Platelet survival Splenectomy

Abstract. Duplicate platelet survival studies, using autologous platelets labelled *in vitro* with radioactive sodium chromate, were carried out on 5 lymphoma patients who had been splenectomized 14–21 months earlier. In the first experiment plasma was employed as the incubation medium and in the second Ringer-citrate-dextrose (RCD) solution. The uptake of chromate by the platelets was 2.0 times higher in the RCD as compared to the plasma experiments. An identical pattern for the immediate behaviour of infused labelled platelets was observed in the duplicate studies, and the recovery of platelet-bound radioactivity remained stable at the 90% level during 2 h after infusion. In these experiments the means for platelet mean life span were almost identical, 5.4 ± 0.6 and 5.3 ± 0.5 days, respectively and significantly ($p < 0.05$) shorter than the mean for control group consisting of 21 healthy males (6.9 ± 0.3 days). It is concluded that RCD and plasma seem to serve equally well as incubation medium at the *in vitro* labelling of platelets.

The *in vitro* labelling of platelets with (^{51}Cr) sodium chromate is currently carried out using plasma as incubation medium. However on the basis of recent studies on the uptake of chromate by the platelets, it was suggested that protein free media should be preferred for the labelling procedure [10, 11] In a study of splenectomized patients with Hodgkin's disease Abrahamsen [2] employed a Ringer-citrate-dextrose (RCD) solution for the incubation. However in those experiments, immediately after infusion, there was a considerable clearance of labelled cells, but after about 15 min they started to reappear in the circulation. On the other hand, this pattern was not observed in a recent study carried out on healthy asplenic subjects in whom the same RCD solution had been em-

ployed [7] In those latter experiments, platelet recovery remained stable around the 90% level throughout 2 h after infusion

The present work was undertaken in order to investigate whether the above RCD solution might harmfully affect platelets of patients with Hodgkin s disease and other lymphoproliferative disorders. The immediate post-infusional fate of labelled platelets and platelet mean life span was determined from duplicate experiments, first using plasma as incubation medium and then RCD

Material and Methods

5 previously splenectomized subjects (3 males and 2 females) were selected for the present investigation (mean age 57 years, range 21–71) The splenectomies had been carried out 14–21 (mean 18) months earlier (table I). 2 of the patients had Hodgkin s disease (nodular sclerosis), 2 had lymphocytic lymphoma, and 1 chronic lymphocytic leukaemia. Patients 1–3 had received repeated courses of combination chemotherapy and subject 5 was treated with chlorambucil (table I).

In all subjects, duplicate platelet survival studies were performed. In the first experiment plasma was employed as the incubation medium and in the second RCD solution [1] The time interval between the two experiments was 6–11 weeks (table I) and the subjects received oral iron supplementation throughout the experimental procedure.

Table I 5 splenectomized patients with lymphomas in whom duplicate platelet survival studies were carried out

Subject No.	Age years	Sex	Diagnosis	Therapy	Time since splenectomy months	Time interval between the two platelet survival studies, weeks
1	21	F	HD-NS	MOPP	17	11
2	66	M	HD-NS	MOPP	21	9
3	64	F	WDL-N	COP	15	7
4	71	M	WDL-N	–	21	7
5	61	M	CLL	chlorambucil	14	6

HD-NS = Hodgkin s disease, nodular sclerosis WDL N = well-differentiated lymphocytic lymphoma, nodular CLL = chronic lymphocytic leukemia MOPP = nitrogen mustard + vincristine + procarbazine + prednisone COP = cyclophosphamide + vincristine + prednisone.

The control group consisted of 21 healthy male volunteers (mean age 37 years, range 22–56) and has been reported on earlier [3]. In all these cases, plasma was used as the incubation medium.

All experiments were carried out using autologous platelets. The entire procedure has recently been described elsewhere in detail [7]. Platelets were labelled *in vitro* using 400 μCi of (^{51}Cr) Na_2CrO_4. The chromium concentration in the incubation medium was 1.73 ± 1.30 SD, range 0.56–4.29 μM. After the infusion of labelled platelets, blood samples were obtained at 5, 10, 15, 30, 45, 60, 90 and 120 min. Thereafter sampling was carried out at 24-hour intervals during a period of 7–9 days [7].

Platelet recovery is the percentage of infused platelet-bound radioactivity in the peripheral blood shortly after infusion and is obtained from: platelet-bound radioactivity $\times$ blood volume $\times$ 100/platelet-bound radioactivity infused. The blood volume was calculated from height and weight measurements [9].

Platelet mean life span was obtained from the zero-time slope of the survival curve [5, 8]. This was achieved by fitting an arbitrary mathematical function

$$Y(x) = Y(0)\left(1 - \frac{x}{T} - A(1-e^{-\alpha x})\right) \quad \begin{cases} 0 < T \\ 0 \leq A \\ 0 < \alpha \end{cases}$$

to the experimental data by the least-squares method using a digital computer. A weight factor of 1 was applied to every experimental point. The mean of activity and time of day-zero 60- to 120-min samples was taken as the first experimental point, and subsequent data were used until about 20% of the initial activity was reached. Y(x) represents PBR at time x after infusion. From the parameters Y(0), T, A and α, Y'(0) was calculated, and platelet mean life span is given by Y(0)/Y'(0). All subjects studied were considered to be in stationary state. Therefore, *platelet production rate* could be calculated from the ratio total number of circulating platelets/platelet mean life span. In asplenic subjects, the total number of circulating platelets is obtained from the peripheral platelet count and blood volume. When calculating the total number of circulating platelets in non-splenectomized subjects, a correction for splenic platelet pooling has to be introduced [6, 7].

The enumeration of platelets was carried out on venous blood using phase microscopy [4]. The platelet counts reported are the values obtained on day zero.

Standard statistical methods were used. Unless otherwise stated, mean values $\pm$ standard error (SE) of the mean are reported. Mean values were tested with Student's t-test. The difference of means was considered significant if $p < 0.05$.

Results

Peripheral platelet count. The means for the peripheral platelet count in the duplicate experiments were $218 \pm 23 \times 10^9$/liter and $230 \pm 30 \times 10^9$/liter respectively. As compared to those values, the control mean was lower ($188 \pm 7 \times 10^9$/liter) but not significantly so (table II).

Table II Results of duplicate (plasma and RCD) platelet survival studies in 5 splenectomiz ed patients with lymphomas and 21 controls

Subject and Incubation media	Peripheral count $\times 10^9$/liter	Recovery %	Mean life span days	Production rate $\times 10^{10}$/day
Plasma				
Case 1	296	93	7 3	14
Case 2	217	94	5 8	17
Case 3	197	88	3 7	20
Case 4	228	80	4 4	28
Case 5	153	85	5.8	15
Mean	218	88	5 4	19
SD	52	6	1 4	6
SE	23	3	0 6	3
Controls, n = 21				
Mean	188	60	6.9	21
SD	34	9	1.2	6
SE	7	2	0.3	1
RCD				
Case 1	332	106	6.1	17
Case 2	256	94	6.5	18
Case 3	200	84	4 0	19
Case 4	201	82	4.5	24
Case 5	159	93	5.2	16
Mean	230	92	5.3	19
SD	67	10	1 1	3
SE	30	4	0.5	1

Initial recovery of infused platelets An immediate clearance of in fused platelet bound radioactivity was not observed in any experiment. As shown in table III the mean platelet recovery remained stable around the 90% level during 2 h after infusion irrespective of whether plasma or RCD had been employed for the incubation procedure. The individual values for platelet recovery obtained on the two occasions given as the mean of 8 consecutive initial determinations are reported in table II. The mean platelet recovery for the plasma study was 88 ± 3%. The corresponding val ue for RCD experiments was slightly higher (92 ± 4%) but these two means did not differ statistically The mean platelet recovery in the con

Table III. Platelet recovery (mean value, SD and SE) during 2 h after infusion obtained from duplicate experiments performed on 5 splenectomized lymphoma patients

Incubation medium	5 min	10 min	15 min	30 min	45 min	60 min	90 min	120 min
Plasma								
Mean	88.8	88.9	87.9	87.8	88.4	89.1	86.9	86.1
SD	5.7	5.2	5 6	6.5	6.1	6.5	6.1	6 0
SE	2.5	2.3	[illegible].5	2.9	2.7	2.9	2.7	2.7
RCD								
Mean	91 7	92.1	91.2	92.7	92.8	92.7	90.9	91.0
SD	8.7	9.6	10.0	10.5	9.8	10.7	9 7	10.2
SE	3.9	4.3	4.5	4.7	4 4	4.8	4.3	4.6

trol group obtained 1–2 h after infusion (60 ± 2%) was significantly ($p < 0.001$) lower (table II)

Platelet mean life span. (Table II). The means for platelet mean life span in the duplicate studies were almost identical, 5.4 ± 0.6 and 5.3 ± 0.5 days, respectively In 1 patient, the results obtained differed by 2.2 days. However in the remaining cases, only minor differences were present. The mean platelet mean life span for the control group (6.9 ± 0.3 days) was significantly ($p < 0.05$) higher As compared to the plasma experiments, the circulating platelet-bound radioactivity in the RCD experiments was considerably (2.0 times) higher

Platelet production rate The individual platelet production rates in the two experiments are given in table II. The means were identical for the two experiments ($19 \pm 3 \times 10^{10}$ and $19 \pm 1 \times 10^{10}$) and did not differ from the control mean ($21 \pm 1 \times 10^{10}$).

Discussion

In the present investigation, no initial clearance of infused platelet bound radioactivity could be noted in any experiment. This is in accord with recent observations made by us on healthy asplenic subjects [7] but at variance with results published by ABRAHAMSEN [2]. This author studied 12 splenectomized patients with Hodgkin's disease, and employed RCD as the incubation medium. During the first minutes, a considerable clearance of PBR was noted, and an average platelet recovery around 60%

was reached 15 min after infusion. Thereafter the circulating platelet bound radioactivity gradually increased and the recovery values obtained 60–90 min after injection were equivalent to those in the present investigation. This apparent difference between the present results and those obtained by ABRAHAMSEN [2] can not be easily explained. The hypothesis that platelets from patients with Hodgkin's disease are slightly injured by the labelling procedure or by the RCD solution [2, 7] is no longer tenable. It seems, however that differences in the time interval between the splenectomies and platelet survival studies in the two investigations could account for the differences in the immediate behaviour of infused PBR observed. Platelets released from the bone marrow during stress might well be more vulnerable to labelling than those formed in steady state, and may therefore undergo transitory sequestration by e.g. the reticuloendothelial system. While all the experiments reported by ABRAHAMSEN [2] were carried out 2 weeks after splenectomy our 2 patients with Hodgkin's disease had been splenectomized not less than 17 months prior to study and in the present series, no experiment was performed earlier than 14 months after splenectomy Therefore, on the basis of our earlier experimental work [7] and the results of the present investigation it can be concluded that RCD does not harmfully affect the platelets.

Platelet survival in the 5 splenectomized lymphoma patients was significantly ($p < 0.05$) shorter than in the control group. The circulating platelet bound radioactivity was shown to be considerably (2.0 times) higher in the RCD as compared to the plasma experiments. The results for platelet mean life span were, however very similar in both experiments. This finding is in accord with our earlier observations made on healthy asplenic subjects [7] and does not lend support to the view that protein-free solutions are to be preferred for the incubation procedure [11] On the other hand, protein free media promote high uptake of chromate by the platelets. Therefore, in the presence of thrombocytopenia autologous platelet survival studies are more easily performed if a protein-free solution is employed instead of plasma.

Patients 1–3 were subjected to intense combination chemotherapy and were all in complete remission at the time of investigation. In none of the 5 lymphoma patients, however was platelet production rate depressed and the mean platelet production rate in the lymphoma group did not differ from the control mean. Thus, as judged from the results of the present work, intense chemotherapy may well be tolerated by lymphoma patients without any discernible depression of the platelet production rate.

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Platelet Antibodies in Different Forms of Chronic Thrombocytopenia[1]

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Key Words. Autoimmune thrombocytopenia · Lupus erythematosus · Platelet antibodies · Serotonin release test · Thrombocytopenia

Abstract. Three techniques have been employed for the *in vitro* detection of circulating platelet antibodies in thrombocytopenic patients affected by 'idiopathic' form or by lupus erythematosus (SLE), the complement fixation test, the platelet *factor 3 availability test and the serotonin release test.* 29 of the 35 sera tested (82.8%) gave positive results for antiplatelet activity. In particular the serotonin release test allows to distinguish 4 groups of patients: a first group affected by idiopathic form, two groups with autoimmune thrombocytopenia and various degrees of serotonin release, and finally a fourth group which comprises subjects affected by SLE, with circulating immunocomplexes.

The conventional tests for antiplatelet antibodies are generally considered of limited diagnostic value, since negative results are obtained in more than 30% of patients with idiopathic thrombocytopenic purpura (ITP). Recently DIXON *et al.* [2] have reported that a new method – the complement lysis inhibition assay – *represents a very useful tool for the* measurement of platelet surface IgG. According to these authors, three main groups of patients with ITP can be distinguished: (1) patients with very high levels of surface antiplatelet antibodies; (2) patients with a slight although significant increase of IgG on the platelet surface; (3) patients without any detectable increase of platelet surface IgG. In addition, a *fourth group might be recognized, formed* by thrombocytopenic patients suffering from systemic lupus erythematosus (SLE) with detectable platelet antibodies.

[1] The technical assistance of Mr. CARMINE CAPITTA is gratefully appreciated.

In the present work, sera from thrombocytopenic patients were tested for the presence of platelet antibodies by three methods: complement fixation test, platelet factor 3 (PF3) availability test and serotonin release test.

Materials and Methods

Sera from 27 subjects with ITP and 8 patients with SLE and thrombocytopenia (cases 6, 12, 16, 18, 24, 27, 28, 29) were tested. Blood samples were taken prior to therapy or at 3-month interval after steroid therapy had ended. Platelet-rich plasma (PRP) was obtained from anticoagulated blood (one part of 3.8% sodium citrate, 9 parts of blood) and centrifuged at 900 rpm for 10 min. Platelet-poor plasma (PPP) was obtained from anticoagulated blood centrifuged at 2,000 rpm for 30 min. Test and control sera were inactivated for 30 min at 56 °C. Guinea pig complement (Behringwerke AG) or human fresh serum from AB-fasting donors was employed as complement source.

Complement fixation test. The microtechnique of platelet complement fixation test according to Colombani *et al.* [1] was employed. The final platelet suspension contained 400,000/platelets/µl. The complement dilution giving two hemolytic units was used for performing the test.

PF3 availability assay. The incubation of normal PRP with thrombocytopenic sera makes PF3 available and therefore the recalcification time is shortened [4]. The reaction mixture (0.10 ml test serum and 0.10 ml PRP) was incubated at 37 °C for 10 min. 0.10 ml of the mixture were added to 0.10 ml PPP; after incubation for 30 min at 37 °C, 0.20 ml $CaCl_2$ were added and the clotting time was evaluated. The reaction was considered positive if the clotting time of the test serum was shortened to at least two standard deviations in comparison with the mean coagulation time of 30 control sera.

Serotonin release test. The platelet serotonin release test depends on the capacity of platelets to release serotonin, as result of antibody attaching to the platelet surface. Platelet suspensions were prepared from blood, anticoagulated with EDTA-Na 10% (0.15 ml/10 ml blood). 10-ml platelet suspensions were incubated with 10 µCi (0.12 mg) of serotonin ^{14}C-bioxalate (Amersham) for 45 min at 37 °C and centrifuged for 10 min at 1,800 rpm. The platelet button was washed three times with balanced buffer protein solution containing 0.5% human albumin (Sigma) [6]. The final concentration of labelled platelets was 400,000/µl. The reaction mixture consisted of 0.05 ml labelled platelet suspension, 0.05 ml test serum i.a., 0.05 ml guinea pig complement diluted 1/27. The final volume was adjusted to 0.20 ml with buffered protein solution. The mixture was incubated in microtiter plate (Cooke Microtiter) at 37 °C for 60 min and then centrifuged at 1,800 rpm for 10 min at 4 °C. 0.05 ml supernatant were adsorbed on a fiberglass disk in counting vial, and dried for 60 min at 180 °C, 10 ml scintillation liquid (PPO 6 g/l and POPOP 10 mg/l toluene), were added to the vial. Radioactivity was measured in a liquid scintillation counter (Mark I, Nuclear-Chicago) for 60 sec. The final result represented the mean value of three determinations and was expressed as counts per minute (cpm).

Table I ^{14}C-serotonin release test (means ± SD of 40 determinations)

Mixture	cpm after incubation at 37 °C for 60 min
Normal platelets + guinea pig's complement 1 27	4 638 ± 534
Normal platelets + guinea pig s complement 1 27 + normal serum	4,563 ± 588
Normal platelets + human complement 1 27 + normal serum	4 622 ± 565
Normal platelets	4,517 ± 660
Normal platelets incubated with triton X 100	28,260 ± 4,214

Table II Platelet counts, platelet complement fixation test and antinuclear antibodies

Case No.	Platelets/µl	Complement fixation test	Antinuclear antibodies
1	90,000	1 2	neg.
2	33 000	–	neg.
3	95,000		neg.
4	5 000	–	neg.
5	70 000	–	pos.*
6	70,000	1 4	neg.
7	66,000	–	neg.
8	80,000	–	neg.
9	85,000	1 2	neg.
10	50,000		neg
11	105,000		neg
12	100 000	1 2	pos.
13	38,000	–	neg.
14	40,000	–	neg.
15	95,000	1 2	neg.
16	100,000	1 2	pos.
17	110,000	–	neg.
18	130,000	1 4	pos.
19	60,000	–	neg
20	90,000	–	neg.
21	80,000	–	neg.
22	60,000	–	neg.
23	80,000		neg.
24	110,000	–	pos.
25	80,000	–	neg.
26	80,000	1 2	neg.
27	140,000	1 8	pos.
28	40,000	–	pos.
29	110,000		pos.
30	100,000	–	neg.
31	90,000	–	neg.
32	110,000	–	neg.
33	80,000	–	neg.
34	90,000	–	neg.
35	55,000		neg.

Spontaneous release of labelled serotonin occurred after incubation of a normal platelet suspension at 37 °C for 60 min (table I) Guinea pig or human complement, as well as normal sera, had no effect on this spontaneous release. Maximum release of radioactivity was obtained when the normal platelets were treated with triton X 100 (Sigma, 0.05 ml of 1/20 diluted solution) Test sera were studied with or

Table III. PF3 availability test

Case No.	Coagulation time sec	Result	Case No.	Coagulation time sec	Result
1	115	neg.	19	115	neg.
2	118	neg.	20	117	neg.
3	130	neg.	21	114	neg.
4	62	pos.	22	79	pos.
5	118	neg.	23	73	pos.
6	116	neg.	24	80	pos.
7	60	pos.	25	60	pos.
8	50	pos.	26	70	pos.
9	120	neg.	27	123	neg.
10	114	neg.	28	119	neg.
11	117	neg.	29	120	neg.
12	119	neg.	30	60	pos.
13	80	pos.	31	117	neg.
14	77	pos.	32	60	pos.
15	77	pos.	33	101	pos.
16	121	neg.	34	119	neg.
17	130	neg.	35	100	pos.
18	62	pos.			

without the presence of guinea pig or human complement. A test serum that caused release of more than two standard deviations was considered as positive.

Antinuclear antibody test. The indirect immunofluorescent technique according to NAKAMURA [7] was employed using unfixed rat liver sections as substrate. The specimens were examined with Leitz Ortholux fluorescence microscope, equipped with Ploem vertical illuminator.

Results

Complement fixation test. This test was negative in all but one patient, in whom a titer of 1:8 was obtained (table II).

PF3 availability assay. The mean coagulation time recorded in 30 normal subjects was 128 ± 7.01 sec. 19 sera gave negative results (table III). 16 sera (45.7%) induced a significant decrease of the coagulation time, to indicate that the PF3 had become available after incubation with these thrombocytopenic sera.

Serotonin release test. Table IV shows the results obtained in thrombocytopenic patients. Three patterns could be distinguished. (1) 11 sera

Table IV ^{14}C-serotonin release test in thrombocytopenic patients

Case No.	^{14}C-serotonin release cpm		Result	Case No.	^{14}C-serotonin release cpm		Result
	without comple ment	with comple-ment			without comple-ment	with comple-ment	
1	8,100	8 700	pos.	19	4 900	4 700	neg.
2	4 700	4 800	neg.	20	3,900	4,900	neg.
3	12,500	12,500	pos.	21	10,900	11 700	pos.
4	6,500	6,500	pos.	[illegible]	12,400	11,900	pos.
5	19 400	20,000	pos.	23	8,570	8,970	pos.
6	18,000	26,000	pos.	24	13 770	23 428	pos.
7	15,770	15,300	pos.	25	4,900	4 400	neg.
8	11 000	13,300	pos.	26	26,330	27 440	pos.
9	10,400	10,300	pos.	27	15,000	30,000	pos.
10	4 700	4 800	neg.	28	13,000	25,000	pos.
11	26,000	26,000	pos.	29	16,000	28,000	pos.
12	8,000	19,500	pos.	30	7,000	8,000	pos.
13	4,300	4,300	neg.	31	4 600	4,900	neg.
14	4 430	4,900	neg.	32	4,900	4,900	neg.
15	15 000	14,300	pos.	33	9,900	9,900	pos.
16	10,790	15,200	pos.	34	4 800	4 700	neg.
17	6,500	6,400	pos.	35	4 600	4,990	neg.
18	8,900	15 770	pos.				

did not induce any abnormal release of serotonin both in the presence or absence of complement (2) 16 sera induced various degrees of serotonin release independently from the addition of complement (3) 8 sera (cases 6 12, 16, 18 24 27 28, 29), which had induced an abnormal serotonin release in the absence of complement, gave rise to a further increase of serotonin release after the additon of complement.

Antinuclear antibody test By the indirect immunofluorescence, 8 of the 35 sera tested gave positive results. These sera were from patients with proven SLE and associated thrombocytopenia (cases 6 12, 16 18 24 27 28 29)

Discussion

Three techniques have been employed for the *in vitro* detection of cir culating platelet antibodies the complement fixation test, the PF3 availa

bility test and the serotonin release test. The first method has proven to be the less sensitive one, since it gave negative results in all but one case. In 5 patients (14.2%) the demonstration of platelet antibodies was achieved by only the PF3 availability test, in 13 patients (37.1%) positive results were obtained by only the serotonin release test. In 11 patients (31.4%) both the PF3 availability test and the serotonin release test were positive. 29 of the 35 sera tested (82.8%) gave positive results for antiplatelet activity to one or more of the methods employed.

The results obtained by the serotonin release test, together with those recorded by the other methods, allow us to distinguish 4 groups of patients with thrombocytopenia: in the first group, all tests done to assess antiplatelet activity give negative results. In the second group, a marked release of serotonin occurs when platelets are incubated with test serum in the absence of complement. In the third group only a slight increase of serotonin release takes place in the absence of complement, and no further increase is seen when the complement is added. In the fourth group a release of serotonin occurs in the absence of complement but a further significant increase, like that induced by triton X 100 occurs when complement is added. Our findings agree with those reported by DIXON and ROSSE [2] and DIXON *et al* [3] who, studying thrombocytopenic patients with SLE, found a significant increase of serotonin release from platelets only in the presence of complement.

The simultaneous performance of multiple tests for the detection of platelet antibodies appears to be of great value towards a better classification of the thrombocytopenic states. The denomination ITP must be confined to a disease in which no platelet antibodies can be detected by the at present available methods. When platelet antibodies are present in the serum, platelet depletion may occur as a consequence of the binding of the antibody to its specific surface antigen. patients in whom such a mechanism operates have to be referred properly as having the classical autoimmune thrombocytopenia

Thrombocytopenia occurs in a certain percentage of patients with SLE, viral infections or drug reactions. The mechanism of platelet depletion in these patients is not fully understood. In the present work we have shown that some sera were able to induce a certain release of serotonin from the platelets in the absence of complement, and that such a release was greatly increased when complement was added. Interesting enough, all sera giving this pattern were from patients with SLE and contained antinuclear antibodies. These findings allow us to speculate about the mech-

anism(s) involved in platelet depletion in SLE. It may be that lupus patients develop platelet antibodies responsible for a classical autoimmune thrombocytopenia According to DIXON and ROSSE [2] platelet antibodies of SLE sera differ from platelet antibodies of ITP sera in that the former are able to fix the complement to the platelet membrane. This observation could account for our findings, but further study is advisable in this area [5] Apart from a direct effect of platelet antibodies, it cannot be excluded that the deposition of circulating immune complexes (such as DNA anti DNA, viral antigen-viral antibody etc.) on the platelet surface, and the related activation of the complement to C8 9 might contribute at a various extent to the platelet destruction in patients with SLE, viral infections or hypersensitivity reactions to drugs.

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Acta haemat. 58 17 2(1977)

Congenital Defi iency of Factor XIII with Normal Subunit S and Lack of Subunit A

Report of Family

Antonio Girolami Alessandro Bu l and Antonio Sticchi

University of Padua Medical School, Instituto f Semeiotica Medica Padua

Key Words Coagulation disorders Factor XIII deficiency Factor XIII subunits

Abstract T sisters born from nonconsanguineous marriage are found to have congenital factor XIII deficiency I the electroimmunoassay system, using an anti-subunit S antiserum, tw distinct peaks or rockets were seen in normal plasma and serum whereas only one peak was present in the propositae plasma or serum. In the bidimensional immunoelectrophoresis system, using the anti-subunit S antiser um, two major peaks were seen in normal plasma whereas only one peak was seen in the propositae plasma. Using an anti-subunit A antiserum no peak or precipitat was seen in our propositae in the electroimmunoassay or in the bidimensional immunoelectrophoresis systems. Both the parents and the children of our two propositae showed normal coagulation pattern. Therefore, the heredity appears t be autosomal recessive. These data indicate that the defect is characterized by normal factor XIII subunit S (support) and lack of factor XIII subunit A (activity)

Congenital factor XIII deficiency has been described by Duckert *et al* [10] in 1961 Since the first description, several other cases have been added. *In 1970, Egbring et al [12] gathered 28 cases, including two per* sonal patients. In the same year Lorand *et al.* [18] published data concerning the inheritance of the condition and referred to 44 patients belonging to 30 families. A few additional cases not considered in such re views have been published before and in 1970, and a few more after that year [., 3 5 15 17] In 1972, Ratnoff and Steinberg [21] gathered 63

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Acta haemat. 58: 17–26 (1977)

Congenital Deficiency of Factor XIII with Normal Subunit S and Lack of Subunit A

Report of a New Family

ANTONIO GIROLAMI, ALESSANDRO BURUL and ANTONIO STICCHI

University of Padua Medical School, Institute of Semeiotica Medica, Padua

Key Words: Coagulation disorders · Factor XIII deficiency · Factor XIII subunits

Abstract. Two sisters born from a nonconsanguineous marriage were found to have congenital factor XIII deficiency. In the electroimmunoassay system, using an anti-subunit S antiserum, two distinct peaks or rockets were seen in normal plasma and serum whereas only one peak was present in the propositae plasma or serum. In the bidimensional immunoelectrophoresis system, using the anti-subunit S antiserum, two major peaks were seen in normal plasma whereas only one peak was seen in the propositae plasma. Using an anti-subunit A antiserum no peak or precipitate was seen in our propositae in the electroimmunoassay or in the bidimensional immunoelectrophoresis systems. Both the parents and the children of our two propositae showed a normal coagulation pattern. Therefore, the heredity appears to be autosomal recessive. These data indicate that the defect is characterized by a normal factor XIII subunit S (support) and a lack of factor XIII subunit A (activity).

Congenital factor XIII deficiency has been described by DUCKERT *et al.* [10] in 1961. Since the first description, several other cases have been added. In 1970, EGBRING *et al.* [12] gathered 28 cases, including two personal patients. In the same year LORAND *et al.* [18] published data concerning the inheritance of the condition and referred to 44 patients belonging to 30 families. A few additional cases not considered in such reviews have been published before and in 1970, and a few more after that year [2, 3, 5, 15, 17]. In 1972, RATNOFF and STEINBERG [21] gathered 63

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Case 2

Our second patient is 33-year-old female who the sister of the prop n. Bleeding manifestations have been mild. The only m or bleeding episodes recal ed by the patient concerned postpartum hemorrhages At he second of these occasi she had to be transfused with 1 U of fresh whole blood No easy bruising w s noted and epistaxis ha only been noted occasionally One tooth extraction was f lowed by mild bleeding only At the time of study the ere no bleeding manifestations.

Material and Methods

Material and methods have been described in deta l elsewhe [13- data will be given herein.

Urea solubility test was carried out by suspending recal fied hole plasm clot in solution of 5 M urea and 1 monocloroacetic acid. Clots in pected every few minutes for the 1st h and subsequently every 2 3 h for t lea 1.

Factor XIII assay was evaluated according t the neutralization te meth o posed by Bohn and Haupt [6] using the reagents supplied by Behring erk l cor tories, Marburg (FRG). The antiserum employed in this assay syst m is an subunit A antiserum. Anti-factor XIII subunit S antiserum, lot 2434H and anti-fa t XIII subunit A antiserum, lot 2421M, were supplied by Behringwerke.

Electroimmunoassay was carried out according to modification of the metho proposed by Laurell [19]. More precisely the test was carried out as follow Behringwerke agarose was dissolved in barbital buffer t pH 8 2, ionic strength 0 03 concentration of 1.2% . Antiserum anti-S was added at concentration of 1.0% whereas antiserum anti-A was added at concentration of 0.3%. The agarose solution was then poured out 10 by 10 cm slides, the thickness of the agar being 2 mm. Three 2.5-mm wells were then punched out one above the other The amount of the plasma introduced in the wells was 16 µl. After 30-min prediffusion period, the field strength applied to the system was 3.5 V/cm for period of about 15 h. The plates were then stained with Coomassie brilliant blue.

The bidimensional immunoelectrophoresis was carried out according to Clarke and Freeman [9] using anti-subunit S antiserum. 15 ml of 1.2% garose solution in barbital buffer pH 8 2, ionic strength 0 03, were poured onto 10×10 glass plate. Using 4-mm well cutter and template, two well were cut. Each well was filled with 20 µl of the normal or test plasma. Contact between the gel and the electrophoresis buffer was established by means of 1-mm thick filter paper strips (Schleicher & Schull No. 2668) The field strength applied to the system was 6 V/cm. Electrophoresis time was about 3 h in a water-cooled apparatus.

Immediately after the first electrophoresis had been completed, gel strips not containing the applied samples were cut with long blade and discarded. 100 ml of 1 2% agarose containing 0.7% anti-S antiserum were poured on the unoccupied area of the plate. This gel sets in 5–10 min, and the plate was then put back in the water cooled apparatus in order to carry out the second dimension electrophoresis. I this electrophoretic run, the direction of the current was at right angle to its direction in the first run. As for the first electrophoretic migration, the containers were filled

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An immunological evaluation of factor XIII antigen is limited so far to a few patients [1 4 8 11 23] Using an overall anti factor XIII antiserum, the presence of normal or slightly decreased factor XIII antigen was demonstrated in the patients so far investigated [1 11 23] This observation could have been interpreted as an indication that factor XIII deficiency was indeed the result of a structural abnormality characterized by a normal antigen protein and by low or absent activity Subsequently biochemical and immunological studies have shown that factor XIII is composed of two units which have been termed subunit S (support) and subunit A (activity) [7 8 18] Using distinct anti S and anti A units antisera, it has been shown in a few cases that these patients seem to have normal subunit S but not subunit A [4 8] This would seem to indicate that the defect in factor XIII deficiency consist in the lack of subunit A synthesis. However the number of patients investigated is still too small to warrant a final conclusion. The possibility of the existence of different molecular abnormalities could always be entertained. The object of the present paper is to report two new patients with this disorder together with an immunological investigation of the condition

Case Reports

Case 1

The propodita is a 30-year-old female who was sent to us for an evaluation of her bleeding tendency in August 1975 Family history was partially positive in the sense that a sister (see case 2) complained of occasional epistaxis and of postpartum bleeding. The parents were not consanguineous. The patient was first noted to bleed excessively after tooth extractions. Menstruation has always been heavy Epistaxis and easy bruising have also been noted in several occasions.

At the age of 19 the patient became pregnant and showed vaginal bleeding throughout the pregnancy In spite of that, the pregnancy was conducted at term but profuse bleeding occurred in the postpartum period On that occasion the patient had to be transfused with 3 U of whole blood with prompt subsidence of the hemorrhagic manifestations. One year later the patient again became pregnant and again showed vaginal bleeding and postpartum hemorrhage On this occasion the patient also had to be transfused. At the ages of 23 and 25 the patient had two other pregnancies which ended in spontaneous abortion accompained by profuse bleeding. Routine coagulation studies resulted to be normal on several occasions.

Case 2

Our second patient is 33-year-old femal who is the sister of the proposita. Bleeding manifestations have been mild. The only major bleeding episodes recalled by the patient concerned postpartum hemorrhages. At the second of these occasions she had to be transfused with 1 U of fresh whole blood. N easy bruising was ever noted and epistaxis has only been noted occasionally. One tooth extraction was followed by mild bleeding only. At the time of study there were no bleeding manifestations.

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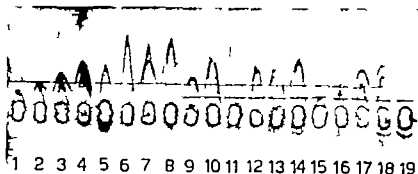
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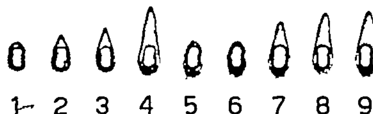


Fig 1 Electroimmunoassay using anti-factor XIII subunit S antiserum 1–4 and 15–18 = 1 8, 1 4, 1 2 and 1 1 diluted pooled normal plasma, 5 = sister of proposita (case 2), 6 = proposita, 7 = proposita daughter 8 = proposita's son, 9 = daughter of case 2, 10 = father of proposita, 11 = serum of proposita, 12 = normal serum 13 = mother of proposita 14 = unaffected sister of proposita 19 = husband of proposita. It is interesting to note that single peak or precipitate is evident only in the propositae plasma and sera Undiluted plasma or serum was used in every instance.

Fig 2 Electroimmunoassay using anti-factor XIII subunit A antiserum, 1–4 = 1 8, 1 4 1 2 and 1 1 diluted pooled normal plasma 5 = sister of proposita, 6 = proposita 7 = niece of proposita 8 = son of proposita, 9 = daughter of proposita. Undiluted plasma was used in every instance.

Table II Solution times of recalcified plasma clots

	5 M urea	1% MCA
Proposita	8 min	5 min
Sister	10 min	6 min
Normal	4 h	4 h

Table I. Coagulation study in our proposita; similar results were obtained in the sister (case 2)

	Patient	Normal values
Clotting time, min	7	5–9
Bleeding time, min	3	<5
Platelet count/µl	250 000	150,000–350 000
Clot retraction	complete	complete in 12 h
Partial thromboplastin time, sec	37.5	32–45
Prothrombin time, sec	14.0	13–14
Thrombin time, sec	19	18–25
Prothrombin consumption, %	>90	>90
Factor II, V, VII, VIII, IX, X, XI, XII	normal	60–160
Factor XIII	see table II	
Thromboelastogram		
r, mm	16	10–20
k, mm	11	6–12
am, mm	4	50–65
Fibrinogen, mg%	360	250–450
Euglobulin lysis time, h	20	10–30

with barbital buffer pH 8.2, ionic strength 0.03. The potential difference applied to the system was 3 V/cm and the electrophoresis time was about 8 h. Plates were then dried and stained with Coomassie brilliant blue.

Results

All routine plasmatic and platelet tests were within normal limits with the exception of the thromboelastogram (table I). In both patients and on repeated occasions, the thromboelastographic abnormality consisted of decreased maximal amplitude and/or an increased postmaximal deflection.

Recalcified plasma clots showed a quick solubility in 5 M urea solution and in 1% monoiodoacetic acid (table II). Specific factor XIII assay using a neutralization test showed consistently low levels of factor XIII in both patients (table III). The electroimmunoassay using an anti S antiserum showed two concentric precipitates or peaks in normal plasma or serum and only one precipitate or peak in the plasma or serum of our two propositae (fig. 1). On the contrary, using an anti unit A antiserum, only one precipitate was seen in normal subjects and no precipitate or peak was

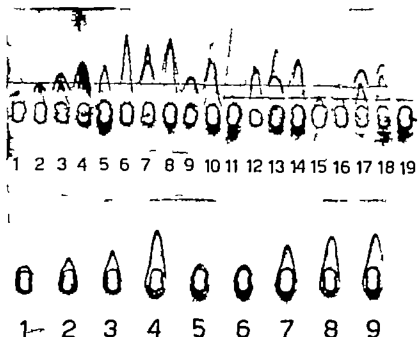


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Table II Solution times of recalcified plasma clots

	5 M urea	1% MCA
Proposita	8 min	5 min
Sister	10 min	6 min
Normal	4 h	24 h

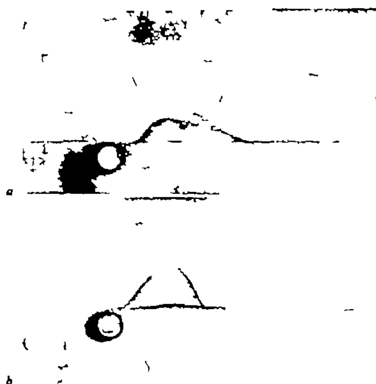


Fig 3 Bidimensional immunoelectrophoresis of proposita's plasma (b) and of pooled normal plasma (a) using an anti factor XIII subunit S antiserum. Only one major precipitate is evident in the proposita's plasma whereas two major peaks are present in the normal plasma. Lighter or minor peaks are evident in both plasmas. These results confirm the electroimmunoassay and suggest that the anti-factor XIII subunit S antiserum is really an an anti-total factor XIII antiserum.

Table III Factor XIII assays in the propositae and in several family members

	Neutralization test of normal	Electroimmunoassay of normal	
		anti-S	anti-A
Proposita, case 1	5	100	0
Affected sister case	5	100	0
Normal sister	110	100	100
Father	100	100	100
Mother	90	100	80
Son of case 1	100	100	100
Daugther of case 1	90	100	100
Niece of case 1	85	100	85
Normal values	70–130	60–160	60–160

noted in our two propositae (fig. 2). In the bidimensional immunoelectrophoresis system, at least two major precipitates and two minor ones were visible in normal plasma whereas only one major precipitate and two minor precipitates were seen in the propositae's plasma (fig. 3)

The study of family members, including the parents and the children of the two propositae showed a normal coagulation pattern. The mother of our proposita and the daugther of her sister showed a factor XIII subunit A antigen level slightly lower than that found in other family members, but still well within normal limits (table III).

Discussion

The main diagnostic criteria for the diagnosis of factor XIII deficiency are normal routine plasmatic and platelet tests as PTT PT TT and bleeding time, defective thromboelastographic pattern, and clot solubility in 5 M urea. Our patients met these criteria fully The specific assay and the immunological studies confirmed the diagnosis.

It is interesting to note that the first suspicion for a factor XIII deficiency came from the thromboelastographic tracing. In the presence of normal fibrinogen and platelets levels, no other clotting disorder could give such pattern. The frequency of thromboelastographic changes in congenital factor XIII deficiency appears to be very high and this observation has somewhat reevaluated the use of this test in routine coagulation studies.

On clinical grounds our propositae did not show any peculiarity Altogether spontaneous bleeding has been mild. After surgical procedures bleeding was severe but no cheloid formation was noted.

The results of immunological studies are of interest. Firstly it seems that the anti-subunit S antiserum used by us is really an anti-total factor XIII antiserum. In the immunoelectroassay a double precipitate is in fact visible in all instances but for the propositae's plasma and serum. These double peaks were always present but the clearness of the image varied. This has important, practical and diagnostic implications since a diagnosis of factor XIII deficiency may be suspected only on the basis of an immunoelectrophoresis carried out with an anti-subunit S antiserum. A single peak or precipitate will be present only in affected members. The possibility that our patients synthetize twice as much subunit S and no subunit A may be ruled out since the level observed in our propositae appears to



Fig 3 Bidimensional immunoelectrophoresis of proposita's plasma (b) and of pooled normal plasma (a) using an anti-factor XIII subunit S antiserum. Only one major precipitate is evident in the proposita's plasma whereas two major peaks are present in the normal plasma. Lighter or minor peaks are evident in both plasmas. These results confirm the electroimmunoassay and suggest that the anti-factor XIII subunit S antiserum is really an on anti-total factor XIII antiserum

Table III Factor XIII assays in the propositae and in several family members

	Neutralization test, % of normal	Electroimmunoassay of normal	
		anti-S	anti-A
Proposita case 1	5	100	0
Affected sister case 2	5	100	0
Normal sister	110	100	100
Father	100	100	100
Mother	90	100	80
Son of case 1	100	100	100
Daugther of case 1	90	100	100
Niece of case 1	85	100	85
Normal values	70–170	60–160	60 160

Addendum

Further studies carried out since the submittal of the paper in these and in other patients with factor XIII defiency have allowed differentiation of factor XIII deficiency in tw groups. The first group is characterized by lack of both subunit S and subunit A. The second group is characterized by normal subunit S and lack of subunit A.

These findings will be reported in future paper

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be similar to that found in a normal subject. The defect seems to consist, in other words in a defective synthesis of subunit A without a compensatory increase of subunit S However the existence of other abnormalities cannot be ruled out. The use of specific antisera in the evaluation of other patients with this disorder is advisable in order to clarify this problem. The presence of two peaks in normal serum suggests that both factor XIII subunits A and S remain in serum after clotting. This is confirmed by the observation that only one precipitate or peak is present in the patients sera.

The electroimmunoassay findings have been confirmed by the bidimensional immunoelectrophoresis The presence in this latter system of more precipitates as compared with the electroimmunoassay is not surprising since it is known that the bidimensional immunoelectrophoresis is a more sensitive method. We have observed a similar phenomenon in a congenital dysprothrombinemia (prothrombin Padua) [15]

The hereditary transmission in our family appears to be autosomal recessive We were unable to differentiate heterozygotes from the normal population Both the parents and the siblings of our propositae showed normal factor XIII activity both immunologically and as activity In other families the heterozygote or carrier state could be separated from the normal population and therefore the hereditary pattern appeared to be in completely recessive [20]

It is likely that both hereditary patterns may occur in congenital factor XIII deficiency The same has been demonstrated in congenital afibrinogenemia and may be interpreted as the result of a different phenotypic expression of the abnormal genes [14] The possibility that a factor XIII evaluation by means of the densylcadaverine method might allow a separation of the heterozygote from the normal subjects seems unlikely but cannot be ruled out. As far as hereditary transmission is concerned, it remains unsolved whether both an autosomal and a sex linked type of factor XIII deficiency exist. This hypothesis was based on the studies of RATNOFF and STEINBERG [21] who showed that consanguinity is more frequent in the families in which females are also affected as compared with those in which only males are affected In our family only females are affected but no consanguinity was present between the parents. The number of families with affected females appears now to be 38 In 22 of these (about 58%), consanguinity was demonstrated The number of families with only males affected stands at 26 and consanguinity is present only in 6 of them (23 %) The difference, as noted by RATNOFF and STEINBERG [21] remains marked and is still unexplained

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Effect of Bleeding on *in vivo* and *in vitro* Colony Forming Hemopoietic Cells

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Key Words. Bleeding · Colony forming · Erythropoietin · Hemopoiesis · Stem cells

Abstract. The effect of bleeding on spleen colony-forming units (CFU-S) and on *in vitro* colony-forming cells with colony-stimulating factor (CFU-C) and erythropoietin (CFU-E) has been evaluated. The *in vivo* and *in vitro* colony-forming cells of the bone marrow show a decrease which for the CFU-E, CFU-C follows a short-lived increase. In the spleen, all progenitor cells assayed have shown a significant and sustained increase.

Erythropoietic regulation appears to be controlled by a feedback mechanism whose major phases are sufficiently clear [4, 6, 8]. The control factors operative at the stem cell level are less well understood. Several studies concerning the behavior of the stem cell compartment under erythropoietic stimuli have appeared [6, 8]. Most of these researches regards the transplantable colony-forming cells in mouse bone marrow or to a lesser degree in mouse spleen and a part deals with the *in vitro* colony-forming cells. As far as we know, no similar study has been carried out on hemopoietic progenitor cells which, in cell culture, form colonies of erythroid cells when the differentiative stimulus is erythropoietin [11].

The studies reported in this paper were undertaken to determine the effect of bleeding, a 'physiologic' stimulus, both on spleen colony-forming units (CFU-S) and on *in vitro* colony-forming cells. The latter were studied both with the colony-stimulating factor (CFU-C) and erythropoietin (CFU-E) as differentiative stimuli. The sequential changes in the bone marrow and spleen CFU-S, CFU-C, CFU-E of mice, simultaneously assayed

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Effect of Bleeding on *in vivo* and *in vitro* Colony Forming Hemopoietic Cells[1]

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Table I Effect of bleeding on some hematological values of C57Bl/C3H mice

	Days							
	0	1	2	3	5	8	12	16
VPRC, %	43	36	36	36	42	43	42	43
WBC/μl	6,300	7 400	5,200	3 800	2,800	3 900	4 500	3 700
Reticulocytes, %	3 7	2.2	3 6	11.8	16.2	9.5	5 5	3 6
Bone marrow cells $\times 10^6$	13 4	16.5	13	13 6	11 4	13 9	12.4	15.4
Spleen weight, mg	76	90	115	152	120	102	90	75

on a same suspension of bone marrow or spleen cells as a function of time after bleeding were followed.

Materials and Methods

Mice were F1 (C57Bl♀/C3H♂), 8–12 weeks old. To check possible variations among different strains, the experiment was repeated on Swiss mice (Charles River) of corresponding age and sex. Mice were bled 0.5 ml (about 30%) of the RBC mass) by orbital sinus puncture in a single phlebotomy Groups of 5 mice were killed 2, 3, 4 8, 12 and 16 days after phlebotomy and matched control groups were simultaneously sacrificed. Spleen weight absolute number of nucleated bone marrow cells in the femur volume of packed red blood cells (VPRC), total leukocyte count and number of reticulocytes per 100 RBC of orbital sinus blood, were determined in all mice with routine methods [5 13].

The technique of marrow collection and determination of bone marrow and spleen cell suspension was the usual one [2, 11 19] Aliquots of the cell suspension were assayed for their content of CFU-S according to the TILL and MCCULLOCH [19] transplant method, of CFU-C according to BRADLEY and METCALF [2] and of CFU E, following the *in vitro* culture described by ISCOVE [11].

The absolute contents of CFU-S, CFU-C and CFU E per femur and per spleen are expressed as a percentage of control values obtained from corresponding groups of untreated mice.

Results

Effect of bleeding on C57Bl/C3H mice (table I fig 1) Erythropoietic activity is enhanced by bleeding as shown by the marked increase of reticulocyte counts. Spleen weight is increased too and reaches a maximum after 3 days. No significant changes are observed in the absolute number of nucleated bone marrow cells. The CFU-S in the femur are sharply de

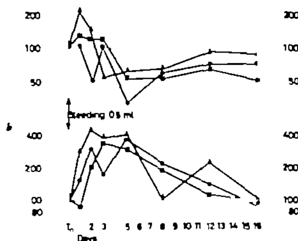


Fig 1 The sequential changes in the bone marrow (a) and spleen (b) CFU-S (●), CFU-C (▲), CFU-E (■) of C57Bl/C3H mice after bleeding. Results are expressed as percentage of control values.

creased as compared with control values on the 3rd to 5th day thereafter their concentration rises but by day 16 the femur CFU S content is not yet restored to control levels. In the spleen, there is a striking increase in CFU-S which reaches a maximum (a nearly 4-fold increase) after 5 days. By day 16, the control content is almost restored.

The number of CFU-E present in the femur rises slightly by day 2 and then shows a significant reduction which persists until day 16. In the spleen, a 2 to 3-fold increase of CFU E is observed, from day 2 almost to the end of the observation time The concentration of CFU-C in the femur first increases, then, after day 2, decreases to 56%. Finally CFU-C reach control levels by day 12. Splenic CFU-C show a striking increase between days 1 and 5 and then decline to control values by day 16.

Effect of bleeding on Swiss mice (table II). The observation period for this group was shorter (8 days) than for the former In general, the effects of bleeding on Swiss mice are comparable to those observed in C57Bl/C3H mice.

Bone marrow CFU-S show an initial decline by days 1 and 2, but thereafter increase to control values. Spleen CFU-S reach 293% of control on day 3 and thereafter remain at levels of over 150% of control.

Table II. Effect of bleeding on some hematological values and on femural and splenic CFU-S, CFU-C and CFU-E (percentage of control values) of Swiss mice

	Days 0	1	2	3	5	7
VPRC, %	40	28	[illegible]3	37	41	4[illegible]
WBC/µl	6,200	7 200	6,200	7 000	5 000	4 300
Reticulocytes, %	4 8	4.2	6.1	8 8	9 6	8 6
Bone marrow cells, × 10⁶	10	9 4	8.5	9	8	9.5
Spleen weight, mg	117	159	161	219	179	127
CFU-S/femur	100	84	66	90	67	85
CFU-E/femur	100	159	187	275	148	66
CFU-C/femur	100	192	206	184	63	33
CFU-S/spleen	100	99	178	293	195	151
CFU-E/spleen	100	198	230	518	322	18
CFU-C/spleen	100	182	140	244	503	318

Bone marrow and spleen CFU E show a striking increase which, in the femur is followed by a rather rapid decline Bone marrow and spleen CFU-C display a pattern similar to that seen with CFU E.

Discussion

The results obtained in the present study are in general accordance with those already reported in the literature. The work of BRUCE and MCCULLOCH [3] BOGGS *et al* [1] MARSH *et al* [13] SMITH and WILLARD [18] HODGSON [10] indicates that in mice subjected to an erythroid sti mulus (hypoxia bleeding, erythropoietin cobalt, testosterone, phenilhy drazine) bone marrow CFU-S are either unaffected or show a slight decrease while usually there is an increase in splenic pluripotential cells. A similar trend of the committed myeloid progenitor cells assayed by the agar colony technique was observed by METCALF [14] and by RENCRICCA *et al* [16] after bleeding phenylhydrazine hypoxia and erythropoietin. Also in the experiment presented here, an increase in the splenic CFU-S and a decrease in the bone marrow CFU S were observed with a pattern which was similar in the two strains of mice checked.

Thus these changes seem to reflect the common response of pluripo-

tential, transplantable stem cell compartments to erythropoietic stimuli. It seems likely that the decrease in bone marrow CFU S is due both to continued differentiation to committed hemopoietic cells and possibly to migration from the bone marrow to the spleen where their increase is already striking in the first days. Migration from the bone marrow to the spleen may reflect a more favorable environment in the latter [12] where expansion of erythropoietic tissue could be physically possible.

The decrease of bone marrow CFU-S may be due to a delay in the onset of their proliferation. As a matter of fact RENCRICCA *et al* [16] observed that the bulk of marrow CFU-S were in cycle only by the 7th day after an erythroid stimulus. On the other hand, aside from possible but unlikely changes in plating efficiency of transplanted bone marrow and spleen cell suspensions, absolute quantitative aspects must be kept in mind. According to METCALF and MOORE [15] the ratio CFU-S in the whole marrow to CFU-S in the whole spleen is 6 1 However it is still possible that the increase of splenic CFU-S does not correspond with the bone marrow decrease. Thus the net effect of bleeding could be an overall decrease in the pluripotential stem cell compartment [15]

After transfusion-induced plethora CFU-S, CFU-C, and CFU-E [9] behaved differently Also in this research, after bleeding, neither bone marrow nor spleen CFU-S changes were found to correspond with those of CFU-E. The number of CFU-E shows a sharp and sustained rise in the spleen and a relatively lesser and short-lived increase in the bone marrow In both sites, CFU-E reach a peak just before the reticulocyte wave that is immediately before the effective erythropoietic response to bleeding. These data seem to be in accordance with those of GREGORY *et al* [9] in that the position of CFU-E on the pathway of differentiation is rather remote from that of the pluripotential stem cell and that CFU-E are quite near to the morphologically recognizable erythroid cells. However passage through these progenitor cells appear to be a necessary step in erythropoiesis.

It is not clear whether the increase in spleen CFU-S after erythroid stimulus is due to proliferation *in situ* or to migration [16] Consequently the splenic CFU-E increase might also be due either to migration of similar cells or of differentiating pluripotential stem cells from the bone marrow or to the proliferation of preexisting pluripotential stem cells. The increase of CFU-E in the bone marrow is slightly less striking than that in the spleen. However here the same considerations made for CFU-S can be made for the absolute number of CFU-E in the bone marrow

If we accept [16] that the bulk of marrow CFU S enters cycle only by day 7 this explains why the increase of CFU E is so short lived On one side, these cells must differentiate to the erythroid line and possibly migrate to replenish the splenic erythropoietic site on the other side for a while, they are not sufficently refurnished by the pluripotential cells.

While the peripheral white blood cells showed a moderate reduction after bleeding, marrow CFU-C, after a quick and very short increase present a 50% reduction splenic CFU-C on the other hand show an increase which lasts until the end of experiment. This pattern bears out the observation of RICHARD *et al* [17] To be able to understand these changes it must be remembered that bleeding not only produces a loss of red cells but also a loss of granulocytes and platelets. According to GAYLOR *et al* [7] after acute hemorrhage, an initial phase of pseudoneutrophilia caused by demargination is followed by an increase in neutrophil production Therefore, it is possible that the changes of CFU-C observed in this and other studies express a real response of myeloid-commited progenitor cells to a specific, albeit feeble stimulus to an increased granulopoiesis. The parallel behavior of CFU-C and CFU E offers scanty evidence that demands for erythropoiesis are met at the expense of myeloid progenitor cells.

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9 GREGORY C. J McCULLOCH, E. A and TILL, J E.. Erythropoietic progenitors

these disorders is a compensatory mechanism, associated with the normal physiologic control of erythropoiesis, or represents an autonomous proliferation of the erythroid marrow. In order to assess these possibilities, the responsiveness to exogenous erythropoietin (Epo) of cultured bone marrow cells from patients with RAHBM was studied. As a result of these studies, the cultured marrow cells could be defined as either responsive or unresponsive to Epo.

Materials and Methods

7 patients with refractory anemia with hyperplastic bone marrow including 5 patients with primary acquired sideroblastic anemia, were studied. The laboratory findings at the time of diagnosis are presented in table I. 5 cases (cases 1–5) with primary acquired sideroblastic anemia had increased iron stores, a significantly elevated percentage of ringed sideroblasts, and, in 4 cases, elevated free erythrocyte protoporphyrin levels. All patients received folic acid; the 5 cases with primary acquired sideroblastic anemia were also treated with pyridoxine. No patient responded to these therapies.

Marrow cells, obtained from these patients or from hematologically normal individuals undergoing evaluation for established malignancies, were cultured, in the presence or absence of Epo, according to MIZOGUCHI and LEVERE's [5] modification of the method of KRANTZ [4]. 5 h before the termination of the culture, 0.5 μCi ^{59}Fe-ferric chloride was added to each dish [5]. At the end of the incubation period, the cells were harvested and newly synthesized radioheme was extracted and quantitated [5]. The stimulation of heme synthesis by Epo was compared with the vehicle-treated control cultures and is presented as percentages above untreated control values.

Results

The synthesis of radioheme by cultured bone marrow cells, obtained from 6 hematologically normal individuals, in response to 0.2 IU Epo per dish was increased 78 ± 15% (mean ± 2 SD) when compared with the vehicle-treated control cultures. The dose of 0.2 IU Epo per plate was adopted after preliminary experiments demonstrated that this amount of Epo produced the optimal stimulation.

On the basis of the reponse of their cultured marrow cells to Epo, the patients can be divided into a responsive an an unresponsive group. In the responsive group, consisting of 3 patients with primary acquired sideroblastic anemia (cases 1, 2, 5) and 1 with refractory anemia (case 7), heme synthesis was enhanced 83, 190, 95 and 82%, respectively by 0.2 IU Epo. In the unresponsive group, consisting of 2 patients with primary acquired sideroblastic anemia (cases 3, 4) and 1 with refractory

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Refractory Anemia with Hyperplastic Bone Marrow Subclassification Based on Responsiveness to Erythropoietin *in vitro*[1]

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Key Words. Bone marrow culture · Erythropoiesis · Erythropoietin · Refractory anemia · Sideroblastic anemia

Abstract. The responsiveness to erythropoietin of cultured bone marrow cells, obtained from 7 patients with refractory anemia with hyperplastic marrow was studied. 5 of these patients' marrows also exhibited sideroblastic changes. Heme synthesis in cultured bone marrow cells was either responsive to stimulation by erythropoietin, or completely refractory. The sensitivity of the bone marrow cells to the hormone was not related to either the clinical or laboratory findings.

Refractory anemia with hyperplastic bone marrow (RAHBM) is a term applied to several disorders of apparently diverse etiology all of which are characterized by chronic anemia associated with either a normocellular or hypercellular marrow and defective red cell production. A subgroup of RAHBM containing a high percentage of ringed sideroblasts is classified as primary acquired sideroblastic anemia. In another group of patients, as well as in some patients with primary acquired sideroblastic anemia, RAHBM probably represents a phase in the development of myeloid leukemia. The pathogenic mechanisms underlying these refractory anemias are not well understood, although abnormalities in the heme biosynthetic pathway have been described in primary acquired sideroblastic anemia [1, 2].

Thus, since RAHBM anemia does not appear to be a single disease entity, it was of interest to establish whether the erythroid hyperplasia in

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ous Epo to stimulate heme synthesis in their cultured bone marrow cells. These observations suggest a pathophysiologic heterogeneity among the patients with RAHBM.

In some cases of primary acquired sideroblastic anemia, the addition of pyridoxal phosphate has been shown to stimulate the activity of δ-aminolevulinic acid synthetase [2]. Although pyridoxal phosphate is present in the culture medium, this cofactor is not able to enhance globin synthesis *in vitro* [7] in reticulocytes obtained from patients with primary acquired sideroblastic anemia. Additionally, none of the patients in our study responded clinically to pyridoxine. This observation suggests that a deficiency in this vitamin plays no role in primary acquired sideroblastic anemia.

GREENBERG *et al.* [3] have reported that the sideroblastic anemias can be divided into two groups according to the colony-forming ability of the bone marrow granulocyte precursor cells in culture. These findings indicate that in some cases of primary acquired sideroblastic anemia there may be abnormalities in cell function or maturation which are more fundamental than those associated with hemoglobin synthesis. The data reported in this communication provide additional support for this hypothesis.

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Table I. Clinical and laboratory data of the patients studied

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Peripheral blood							
Age	75	87	72	87	63	46	68
Sex	M	F	F	M	M	M	M
Hb, g/dl	7.9	9.5	8 4	8.8	8.2	5.2	10.1
Hct, %	24 7	30.0	24.0	27 0	26.1	17 0	32.0
Reticulocytes, %	0.8	21.0	ND	1.2	1.2	0.8	1 3
MCV μm³	85.5	79 0	ND	101 0	89 0	78.0	98 0
MCH pg	26.0	23 1	ND	32.6	27 6	26.2	31 6
MCHC, %	30 4	29.2	ND	35 6	31 7	39.8	35.0
Fe, μg/dl	77	65	125	180	145	55	64
TIBC, μg/dl	138	170	200	230	245	40	220
WBC/μl	6 600	5 100	6,300	4,200	4 100	3 600	2,500
Bone marrow							
Cellularity	increased	normal	normal	increased	increased	increased	normal
M/E ratio	0.5/1	2.1/1	ND	1/1	1.5/1	1/9	1/1 1
Erythroblasts, %	65	38		53	45	88	46
Myeloid maturation arrest	–	–	–	–	+	–	+ +
Iron stores	+ + +	+ + + +	+ + +	+ + + +	+ + + +	+ + + +	+ +
Ring sidero-blasts, %	75	66	45	63	56	1–2	1

ND = Not done.

anemia (case 6) the marrow cells were completely refractory to stimula tion by Epo

The bone marrow responsiveness to Epo appeared unrelated to hema tocrit, red cell indices, serum iron and/or total iron-binding capacity Moreover marrow erythroblast percentage and maturation, the presence of myeloid abnormalities, or the percentage of ring sideroblasts was also unrelated to the marrow response to Epo

Discussion

In the present study the patients with RAHBM irrespective of the presence or absence or ringed sideroblasts, could be divided into a responsive and an unresponsive group on the basis of the ability of exogen

ous Epo to stimulate heme synthesis in their cultured bone marrow cells. These observations suggest a pathophysiologic heterogeneity among the patients with RAHBM.

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Waldenström Like Immunocytic Lymphoma with IgG Serum M Component

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Key Words. IgG serum · Immunoproliferative disorders · Lymphocyte IgG · Lymphocyte markers · Lymphoma · M component · Waldenström's disease

Abstract. A case of immunoproliferative disorder with clinical features of Waldenström's disease but with an IgG k instead of IgM serum M component is described. The lymphocyte population in the bone marrow, blood and lymph nodes was studied by rosette test, culture with PHA and immunofluorescence staining. Most of the bone marrow lymphocytes had membrane IgG. This case represents an intermediate form between myeloma and Waldenström's disease, thus supporting the unitarian concept of all the immunoproliferative disorders.

Since Dameshek [2] introduced the concept of immunoproliferative disorders to encompass all the disorders of the plasma cell and lymphocyte proliferation associated with serum M components, several new types of such dyscrasias have been described, some of which represent intermediate forms between the previously recognized entities, namely myeloma, Waldenström's disease, heavy chain disease (HCD) and primary amyloidosis. In this paper we describe a case of an hitherto unrecognized form characterized by clinical and pathological features of Waldenström's disease associated with the presence of a large amount of monoclonal IgG-k immunoglobulin in the serum.

Case Report

R. C., 57 years old, a Piedmontese farmer, enjoyed good health until April 1972 when he began to complain of weakness, fatigue and abdominal distension. In March 1973 he had an abundant epistaxis with severe weakness, blurred vision, conspicuous splenomegaly, and therefore he was sent to hospital.

On admission he was found to be anaemic (Hb 8.5 g%), leukopenic (WBC 4,400/µl with 52% neutrophils) and thrombocytopenic (89,000/µl). Sternal marrow was hypocellular: myeloid series were depressed, whereas there was an increased number of lymphocytes; plasma cells were very few. Biopsy of the iliac crest was normal. Serum proteins were 10.5 g%; electrophoresis revealed a peak in the gamma zone which was identified as an IgG with k light chains. Radial immunodiffusion gave the following values: IgG 8,000 mg%, IgA 27 mg%, IgM 33 mg%. Serum immunoelectrophoresis did not reveal fragments of heavy chains or isolated light chains. Bence Jones protein was not found in 20-fold concentrated urine. Skeletal X-ray was negative. The patient underwent blood transfusions and was treated with prednisone, ACTH and cyclophosphamide. General condition improved and splenomegaly decreased slightly. From May 1973 until September 1974 the patient did well. In September 1974 anaemia relapsed. Prednisone and ACTH were given again until December 1974. In March 1975 the patient was admitted to our hospital. He was severely anaemic and could hardly walk because of profound weakness and of paresthesia to the lower limbs. Oozing from gums and nose as well as petechiae on the legs were present. Axillary and inguinal lymph nodes were enlarged, splenomegaly was huge, reaching the iliac fossa.

Hb 7.2 g%; WBC 1,900/µl with 72% lymphocytes; platelets 30,000/µl. ESR 113 mm/h. Serum proteins 11.2 g% with 64.2% γ-globulins. Immunoelectrophoresis: monoclonal IgG-k component. Cryo-globulins absent; Bence-Jones protein absent. Hepatic and renal function tests normal. Skeletal X-rays negative. Spleen scanning: uneven distribution of the tracer within the greatly enlarged spleen. Abdominal lymphangiography: enlargement and uneven opacification of the ileopelvic and lumboaortic lymph nodes.

Biopsy of a right inguinal lymph node: reactive hyperplasia without other pathological features. Sternal and iliac crest marrow hypocellular with 70% small lymphocytes, 10% intermediate cells, very few plasma cells. The patient was given two courses of melphalan 0.2 mg/kg/day and prednisone 50 mg/day for 5 consecutive days interspersed by a 40-day rest period. 1 week after the end of the second course the patient's condition was almost unchanged, so we reverted to cyclophosphamide and prednisone which produced a remarkable improvement of the general condition and the decrease of the splenomegaly to about one half the pretreatment size. In December 1975 he had herpes zoster which was treated with ARA C with remarkable success. Following this treatment, a further decrease of splenomegaly was observed. The patient is now still anaemic, leukopenic and thrombocytopenic but does not require blood transfusions to keep haemoglobin level above 9 g%. Serum proteins are still 11 g% but albumin is increased by 50% and γ-globulins are decreased from 64 to 50%. The patient feels well and can attend his work.

Final diagnosis: Waldenström-like lymphoma with IgG-k serum M component.

Material and Methods

Peripheral blood lymphocytes (PBL), bone marrow lymphocytes (BML), and cell suspension of a groin lymph node were investigated.

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Table I. B and T markers on lymph node lymphocytes, bone marrow lymphocytes and peripheral blood lymphocytes in patient and controls

	IgG %	IgM %	PHA %	E rosettes %	EA rosettes %	EAC rosettes %
Lymph node lymphocytes						
Patient	6	0	35	15	0	0
Controls	25±5	18±3	60±5	58±8	17±5	22±4
Bone marrow lymphocytes						
Patients	40		0	0	0	5
Controls	0.2±0.1	0.5±0.1				
Peripheral blood lymphocytes						
Patient	6	4	40	2[illegible]	10	10
Controls[1]	12±2	10±3	65±5	63±8	20±5	20±7

Mean value ± standard deviation.

Lymph node. The inguinal lymph node had some modifications of the architecture: rare follicles with germinal centres located in the cortical site were present. On the other hand, paracortical area was prominent. In this site some plasma cells were also found. An ultrastructure picture of the lymph node did not show significant modification.

Immunological tests. All the results are summarized in table I. The percentages of Ig bearing lymphocytes were 6% with IgG and 4% with IgM in peripheral blood, 40% with IgG and 2% with IgM in the bone marrow, 6% with IgG and 0% with IgM in the lymph node. After trypsinization, IgG fluorescence was found to reappear after 5 h on the lymphocyte surface in a percentage similar to that observed in untrypsinized cells. The PHA blastic transformation of PBL was 40%, while the rosetting lymphocytes were 22% for E, 10% for EA and 10% for EAC. Neither B and EA rosetting nor PHA responsive lymphocytes were found in the bone marrow but 5% of cells were positive for EAC. On the contrary in the lymph node EA and EAC rosettes were absent. Only 15 of E rosettes and 35% of blasts in PHA culture were counted. In table I there are also reported immunological data obtained in many subjects without immunological disorders and considered as controls.

Light microscope A part of a lymph node was fixed in buffered formalin and stained with haematoxylin and eosin.

Lymphocyte separation. 10 ml of heparinized peripheral blood, the bone marrow specimen and the lymph node cell suspension were diluted in phosphate-buffered saline (PBS) and layered on Ficoll-Urovision. After a centrifugation at 200 g for 30 min the lymphocytes were isolated and washed twice with PBS. An aliquot of the cell suspension was tripsinized and examined immediately and after 1 and 5 h according to PREUD'HOMME and SELIGMANN [10]

Immunofluorescent staining The lymphocytes to test were incubated at 4 °C for 30 min with anti-human IgG and anti-human IgM fluorescein-conjugated rabbit sera. The anti-γ-globulins (Behringwerke) were used undiluted. After two washes the cells were placed on a slide and examined with a Leitz microscope equipped with an HBO high pressure mercury lamp.

PHA blastic transformation. The cells obtained from the peripheral blood were stored in TC 199 Wellcome with antibiotics and autologous serum. All cultures were performed both with and without addition of PHA (0.02 ml/ml) After 72 h at 37 °C a morphological evaluation of the blast transformation was carried out.

E. rosettes. Sheep erythrocytes (SRBC) previously washed in PBS were mixed with the lymphocyte suspension to test in the ratio of 8–10 SRBC to 1 lymphocyte. The mixed cell population was centrifuged at 200 g for 15 min and then incubated at 22 °C for 60 min. A drop of the cell suspension stained with a brillant cresyl blue solution (Merck), to assess cell viability was placed in a Bürker chamber and the total number of lymphocytes and E rosettes with 3 or more SRBC attached to their membrane was counted.

EA rosettes. EA were obtained by incubating an equal volume of E suspension and rabbit anti-SRBC globulin (Sclavo) diluted 1:8,000 for 30 min at 37 °C. After washing, the EA complexes were mixed with the suspensions to test as previously described, and incubated at 37 °C for 30 min.

EAC rosettes. EA complexes were incubated with an equal volume of human complement diluted 1:20 at 37 °C for 30 min and mixed, after two washes, with the lymphocytes. The ratio between the two cell populations, the incubation of the mixed cell suspensions and the evaluation of the percentage of EAC rosettes were carried out according to the same procedure as done for EA and E rosettes.

Electron microscopy The material for electron microscopy was fixed in buffered 3% glutaraldehyde, postfixed in 1 / osmium tetroxide and embedded in araldite (Durcupan ACM) Thin sections obtained from an LKB Ultrathome ultramicrotome were stained with lead citrate and uranyl acetate, and observed under a Siemens El miskope I electron microscope.

Results

Bone marrow The material was not abundant. Lymphoid cells were prevalent and few plasma cells were also observed. Ultrastructure features of the lymphoid cells were predominant those of the small lymphocytes some cells showed transitional aspects typical plasma cells were seen only occasionally

and VITETTA [13] clearly demonstrated that B-lymphocytes not only produce surface immunoglobulins but also secrete minute amounts of Ig, mainly IgM. Precursor B-lymphocytes display only IgM and IgD [8] on their surface. PIERCE *et al.* [9] showed that under antigenic stimulation a shift occurs in surface immunoglobulin of lymphocytes from IgM to IgG and IgA, a shift which is mediated by T-lymphocytes further antigenic stimulation induces the transformation of B-lymphocytes into plasma cells which produce large amounts of Ig.

If the proliferation of a neoplastic clone of B-lymphocytes is the basic event in myeloma as well as in Waldenström's disease, no wonder that under peculiar circumstances the proliferating lymphocyte may undergo the shift in the surface immunoglobulin from IgM to IgG thus becoming an IgG producer without proceeding to the stage of plasma cell. In our case the intense lymphocyte proliferation and, consequently the huge splenomegaly and the generalized involvement of lymph nodes and bone marrow could account for the production of a relatively large amount of IgG

The hypothesis could be put forward that in immunoproliferative disorders the type of cellular proliferation and the class of immunoglobulin produced may depend more from environmental factors than from intrinsic characteristics of the neoplastic cell. Thus the absence of osteolytic lesions in Waldenström's disease, in HCD as well as in our case, might be explained by the absence of an antigenic stimulation capable of inducing, on the one hand, the transformation of lymphocytes into plasma cells and, on the other hand, of the production of the osteoclast stimulating factor that MUNDY *et al.* [7] have shown to be produced by myelomatous cells as well as by lymphocytes under mitogen stimulation *in vitro* The case we have reported here may represent a missing ring of a chain of clinical situations which could be forecast theoretically and provides further evidence to the unitarian concept of all immunoproliferative disorders as a consequence of the proliferation of a neoplastic clone of lymphocytes, the clinical expression of which is modulated by a number of factors still to be identified.

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Discussion

Huge splenomegaly generalized involvement of lymph nodes, lymphocytic transformation of the bone marrow blood lymphocytosis, long clinical course without osteolytic lesions, and renal impairment are typical features of lymphomas and are found in Waldenström's disease which is included among the low grade malignancy lymphoplasmocytoid lymphomas by LENNART *et al* [5] Monoclonal immunoglobulins may be found in the serum of patients with lymphosarcoma and lymphatic leukemia [1 11 12] and are always present in Waldenström's disease [14] However at variance from Waldenström's disease, in our case the serum M component was an IgG instead of an IgM immunoglobulin and its amount was far much greater than in all previously reported cases of lymphoma. Indeed, such a great amount of monoclonal IgG is encountered only in multiple myeloma however the clinical picture of this disease is usually quite different. Gamma HCD was ruled out by the absence of fragments of heavy chains Our case resembles closely that described by ISOBE and OSSERMAN [3] in which, however gamma heavy chains and free lambda chains were present beside IgG lambda molecules. Moreover in ISOBE and OSSERMAN's patient bone marrow was frankly plasma-cellular We can therefore conclude that this is a case of an hitherto undescribed variety of immunoproliferative disorder characterized by a clinical and haematological picture of Waldenström's disease with an IgG myeloma like serum protein pattern.

Investigation of the lymphocyte populations provides some useful informations as it revealed a conspicuous increase of IgG bearing B-lymphocytes in the bone marrow We did not carry out immunofluorescence staining of lymphocytes with idiotypic antisera against myelomatous protein, therefore we cannot state with certainty that the bone marrow lymphocytes actually produced the homogeneous serum protein component. However indirect evidence is provided by the overwhelming predominance of lymphocytes with membrane IgG in the marrow

A rigid separation between lymphoproliferative (Waldenström's disease, CLL) and plasmaproliferative (myeloma) disorders is not justified In every type of B immunoproliferative disease the cell affected by neoplastic mutation is always a B-lymphocyte, irrespective of its morphological or functional status, as shown by MELLSTEDT *et al.* [6] who, in the blood of patients with myeloma, found a monoclonal population of lymphocytes with the same idiotype of the myelomatous plasma cells. UHR

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Increased Serum Folate-Binding Capacity

A Familial Trait

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Key Words. Radioassay · Serum folate · Serum folate binding protein

Abstract. This report describes increased folate-binding capacity (FABC) in the serum of 7 of 10 family members. Genetically controlled elevation of FABC in normal subjects needs to be distinguished from the acquired increase in binding capacity which has been described in a number of disorders.

Introduction

Ligand radioassay methods have been applied successfully to the determination of serum folate levels [1, 4, 7, 11, 12, 16]. However, the presence of unsaturated serum folate-binding protein (FABP) which binds exogenous folate may significantly affect the serum folate level obtained by radioassay [5, 10, 17, 18, 22]. Increased serum folate-binding capacity (FABC) has been described in some normals and in a number of conditions including folate deficiency [12, 17], pregnancy and the administration of oral contraceptive agents [3] and diphenylhydantoin [8], as well as in multiple myeloma [8], azotemia [2, 6], cirrhosis [2] and pernicious anemia [9]. This report describes increased serum FABC appearing as a familial trait which could not be attributed to previously described conditions associated with increased FABP.

Materials and Methods

Radioassays for serum folate, utilizing a partially purified milk folate binder were performed according to the method of Rothenberg et al. [12] with the excep-

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Increased Serum Folate-Binding Capacity

A Familial Trait

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Increased Serum Folate-Binding Capacity

A Familial Trait

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Key Words. Radioassay · Serum folate · Serum folate binding protein

Abstract. This report describes increased folate-binding capacity (FABC) in the serum of 7 of 10 family members. Genetically controlled elevation of FABC in normal subjects needs to be distinguished from the acquired increase in binding capacity which has been described in a number of disorders.

Introduction

Ligand radioassay methods have been applied successfully to the determination of serum folate levels [1, 4, 7, 11, 12, 16]. However, the presence of unsaturated serum folate-binding protein (FABP) which binds exogenous folate may significantly affect the serum folate level obtained by radioassay [5, 10, 17, 18, 22]. Increased serum folate-binding capacity (FABC) has been described in some normals and in a number of conditions including folate deficiency [12, 17], pregnancy and the administration of oral contraceptive agents [3] and diphenylhydantoin [8], as well as in multiple myeloma [8], azotemia [2, 6], cirrhosis [2] and pernicious anemia [9]. This report describes increased serum FABC appearing as a familial trait which could not be attributed to previously described conditions associated with increased FABP.

Materials and Methods

Radioassays for serum folate, utilizing partially purified milk folate binder, were performed according to the method of Rothenberg et al. [12] with the excep-

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Table I. Serum folate levels obtained with milk and serum binders

Patient	Milk binder ng folate	Serum binder ng folate
1	18.5	4.0
2	13.2	1.6
3	4.2	0.6
4	2.1	0.5
5	0.0	0.1

Effect of heat and storage of FABC. Sera from family members with normal FABC showed a 99–100% reduction in FABC upon heating at 56 C for 60 min while sera from family members with initially increased FABC demonstrated a decrease in binding capacity by 70–76%. Storage of the propositus' serum for 1 year frozen at –20 C did not diminish serum FABC.

In vivo saturation study. The propositus was given 5 mg folic acid orally to test *in vivo* saturation of the serum binder. Rapid saturation of the serum binder was evident over the initial 4 h, accompanied by a rapid rise in serum folate levels. After 6 h there was progressive reappearance of FABC in the serum until baseline levels were reached at 53 h.

Milk and serum binders in folate assay. Table I tabulates the results of serum folate assays on sera tested against the standard milk binder and the propositus serum binder. Consistently reduced binding capacity was shown for the serum binder.

Discussion

Recent reports have described a variety of conditions associated with increased unsaturated FABP which may interfere with the determination of serum folate levels [2, 3, 6, 8, 9, 12, 17]. In the present study FABC greater than 5% was detected in 5.4% of 277 hospitalized patients. In contrast, Zettner and Duly [23] have found elevated levels of unbound FABC in 25% of normal sera and in a somewhat larger fraction of hospitalized patients. Variations in the radioassay methods employed by different workers may well explain the differences in folate binding observed [13, 15, 19].

The presence of increased FABC in the serum of a healthy laboratory

tion that ascorbic acid was not used for preservation of serum folate. High specific activity ^{3}H-pteroylmonoglutamic acid (^{3}H PGA), 52 Ci/mm (Amersham Searle, Arlington Heights, Ill.) was used as the tracer and N^5 methyltetrahydrofolic acid (Methyl FH_4) (Sigma Chemical Company St. Louis, Mo.) as the standard. The milk folate binder was diluted 1:200 with 20% buffered folate-free serum (charcoal adsorbed). Maximum binding of the ^{3}H PGA by the binder was in the range of 35–40%. Serum FABC was determined from the 'serum blank' which contained 20 µl of patient serum, 0.38 ml of buffer 200 pg of ^{3}H PGA but no milk binder. Folate-binding capacity was expressed as the percent of total counts of ^{3}H PGA added (200 pg).

Results

Patients. Serum folate radioassays were performed on 277 hospitalized patients. Of these, 230 sera (83.0%) revealed normal folate levels (greater than 3 ng/ml) 32 sera (11.6%) gave deficient values (0–3 ng/ml) and 15 patients (5.4%) demonstrated increased FABC. Previous studies in this laboratory have shown good correlation between this radioassay method and the *Lactobacillus casei* microbiologic assay [11]. Increased FABC was defined as binding greater than 5% of the total ^{3}H PGA added.

Family study. Ten family members in three generations were studied (fig. 1). All members of the family studied were in good health had no history of anemia, and were not receiving folic acid or oral contraceptive medication. The propositus father and both living siblings along with 3 out of 4 of her sister's children were found to have increased FABC in their sera. The propositus mother and brother in-law had normal FABC, while one niece (III 3) demonstrated high normal FABC.

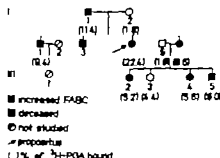


Fig. 1. Family tree illustrating family members with percent ^{3}H PGA bound (FABC) in parenthesis.

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technician stimulated further search for the presence of increased FABC in members of three generations of this individual's family (fig 1). 7 of 10 family members demonstrated increased FABC. These findings are compatible with a dominant nonsex linked inheritance

Previous studies have shown that FABP can be destroyed by heating [7] Heating destroyed 70–76% of the increased FABC present in affected family members. The binding of ³H PGA to FABP *in vitro* has been characterized as a saturable process with a rapid association and a slow dissociation rate [22] In our study early saturation of FABC and a delayed rise in serum folate levels were observed *in vivo* following the administration of oral folic acid. FABP present in serum and milk have shown differences in their ability to bind folates [14 17 20 21] In our hands the propositus serum binder demonstrated diminished binding to serum folate over the standard milk binder (table I) An increased specificity of the serum binder for PGA would allow other folates to remain largely unbound and therefore available to tissues.

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Biochemical Studies on the Leukocytes in Chediak-Higashi Syndrome[1]

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Key Words. Chediak-Higashi syndrome · Enzyme release from leukocytes · Exocytosis · Ionophore A23187 · Leukocyte granules · Myeloperoxidase

Abstract. Blood leukocytes from a patient with Chediak-Higashi syndrome (CHS) were compared with normal cells for their capacity of extruding (exocytosis) the lysosomal enzyme myeloperoxidase during phagocytosis or after a treatment with the ionophore A23187 and Ca^{++}. A decreased rate and extent of exocytosis in phagocytizing CHS cells was observed also with the Ca^{++} ionophore. This suggests that a defect in Ca^{++} mobilization is not responsible for the impaired secretion of granule content. Isolated granules of CHS cells and of leukocytes were treated with the detergent Triton X 100. Since the solubilization of myeloperoxidase from the CHS granules was much lower than from the normal ones, we suggest that the former organelles have a more resistant membrane.

The increased susceptibility to infections of subjects with Chediak-Higashi syndrome (CHS) has been ascribed, besides to neutropenia [4], to a number of functional defects of granulocytes. According to CLARK and KIMBALL [5] and CLARK *et al.* [6], CHS leukocytes have a decreased responsiveness to chemotactic stimuli. Furthermore, ROOT *et al.* [18] have shown that CHS leukocytes, although exhibiting a normal rate and capacity of phagocytosis, have an impaired bactericidal activity. Biochemical and electro-microscopic studies have proved that this impairment is linked to a defective discharge of bactericidal factors from the giant lysosomes of the CHS granulocytes into the phagocytic vacuoles [11, 18, 20].

[1] This work was supported by grant No. 75.00695.04 from the National Research Council of Italy.

The process of extrusion of the granule content into the vacuoles (exocytosis) involves a number of steps, such as elaboration of the exocytotic stimulus at the level of the plasma membrane [19, 23], activation of microfilament systems for granule translocation [1, 21, 24] and finally fusion between the membrane of granules and phagocytic vacuoles.

The activation of the granule translocation system and the fusion process [2, 13] are very likely triggered by ionized calcium which in both phagocytic and non-phagocytic cells is thought to provide the coupling between stimulus and secretion [7]. Thus the defective exocytosis in CHS leukocytes might reside in a functional alteration of cell structures such as cytoskeleton components or granule membrane and/or in an impaired mobilization of intracellular Ca^{2+}.

We have investigated the latter possibility by using the ionophore A23187 which can catalyze the transport of Ca^{2+} from the extracellular fluid toward the cytosol down to its concentration gradient [14]. Since the extrusion of granule enzymes into the extracellular fluid is an indirect measure of intravacuolar exocytosis, we have followed the effects of A23187 by assaying the selective release of myeloperoxidase (MPO) from intact granulocytes. Furthermore, with the aim of obtaining some information on the structural properties of the CHS lysosomal membranes, we have compared the pattern of solubilization of MPO from isolated normal and CHS granules after their exposure to the non-ionic detergent Triton X 100.

Case Report

The patient is a girl whose weight at birth (1968) was 2.77 kg. Her parents are not related; she has 2 sisters and [illegible] brothers in good health. At 6 months of age she presented whooping cough; afterward she started having frequent respiratory tract infections and was frequently hospitalized for treatment of bacterial bronchopneumonia, otitis and abscesses.

In December 1970, she was admitted to the pediatric clinic of the University of Padova where the diagnosis of CHS was made. In April 1974, she was referred to the pediatric clinic of the University of Trieste for further investigations. On admission to the hospital her weight was 18 kg and the height 105 cm. Her hair was light in color. She exhibited photophobia, bilateral horizontal nystagmus, hypopigmentation of irides and retinae. Her liver and spleen were palpable 2 and 8 cm below the costal margin, respectively.

Hemoglobin 11.2 g%, WBC 5,400/µl with 1[illegible]% neutrophils, 80% lymphocytes and [illegible]% monocytes. Platelet count was within normal limits. The bone marrow revealed normal cellularity; all series of WBC showed large inclusion bodies. Serum immunoglobulin levels (mg/100 ml): IgG 1,500, IgA 190, IgM 420. The delayed skin

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Solubilization of MPO from isolated granules. Granules were separated from homogenates of control and CHS cells as previously described [12]. Briefly homogenates in 0.34 M sucrose (pH 7) were first centrifuged at low speed to remove nuclei and unbroken cells and then at 20,000 g for 20 min to precipitate the granules which were resuspended in 0.34 M sucrose. All the procedures described were carried out at 0–4 C. Granules derived from 1 3 10⁸ cells were incubated at 37 °C in the presence of 0.005% Triton X 100 (vol/vol) for 5 min and rapidly centrifuged at 19,000 g for 2 min in an Eppendorf centrifuge (Zentrifuge 3200). The supernatant was carefully withdrawn and assayed for peroxidase activity. Total peroxidase activity of the granules was determined by preincubating the granule fraction in the assay medium at 37 °C with 0.02% Triton X 100 for 3 min before adding hydrogen peroxide.

Chemicals. Latex beads from Difco (0.81 μm) were washed twice in isotonic NaCl and then resuspended at concentration of 30 mg/ml. The spherule concentration was measured as described by ROBERTS and QUASTEL [15]. Ionophore A23187 generous gift of Dr. HAMILL (Ely Lilly Co.), was dissolved in dimethyl sulfoxide. $CaCl_2$ and Triton X 100 were of reagent grade.

Results

Morphology of CHS leukocytes. Electron microscopy of CHS leukocytes confirms the presence of typical giant lysosomes as previously described by others (fig. 1). Virtually all the peripheral neutrophils, and sometimes also the mononuclear cells, have anomalous organelles although of different size in the cytoplasm. Giant lysosomes are easily identified also by light-microsopic examination of May-Grünwald- and Giemsa-stained smears of the white cell population. Differential counts of the isolated white blood cell population show a lower content of neutrophils in the CHS population (35 /) than in the normal one (70%)

Phagocytosis and metabolic stimulation. Whole blood leukocytes of the patient ingest heat-killed *S. aureus* at the same rate as control cells. Furthermore, upon a challenge with latex beads the CHS leukocytes undergo the same enhancement in ¹⁴CO yield from 1-¹⁴C-glucose as observed with normal cells. These results confirm previously reported data [18]

MPO release from leukocytes during phagocytosis. Table I shows the MPO release from normal and CHS leukocytes at different times of incubation. Phagocytizing CHS cells release the granular enzyme at a lower rate than the phagocytizing control cells. In particular at 10 min of incubation phagocytizing normal cells release extracellularly about 50% of the enzyme whereas no MPO is extruded from CHS cells. As indicated by the leakage of LDH MPO released at later time may be partially due to

test reactions to PPD candida and tetanus toxoid were negative. The reponse to DNCB stimulation was normal. The Rebuck skin window showed a normal cellular pattern. The lymphocyte stimulation with PHA determined by ^{3}H thymidine incorporation was normal.

Methods

White blood cell collection. Leukocytes were separated from whole blood as previously described [23]. Briefly, peripheral blood from the patient and healthy subjects with the same age was collected in ACD solution (4:1). leukocytes were isolated after dextran sedmentation of the erythrocytes and freed from contaminating red cells by hypo-osmotic lysis (60 sec in 0.2% NaCl). The leukocytes were then resuspended in a medium containing (mM) 123 NaCl, 5 KCl, 1.2 $MgCl_2$, 0.2 glucose, 16 Na/K phosphate buffer at pH 7.4 (Krebs-Ringer phosphate: KRP) or in a medium containing (mM) 123 NaCl, 5 KCl, 0.2 glucose, 16 Tris-HCl buffer at pH 7.4 (KRT).

Light and electron microscopy techniques. Conventional May-Grünwald-stained smears of control and CHS white cell suspensions were prepared for direct examination of cell morphology. For electron-microscopic observation, cell suspensions were fixed by mixing with equal volumes of chilled 6% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) containing 0.06 M sucrose. After 90 min at 4 °C, they were pelleted by centrifugation. The pellet was rinsed with 0.1 M cacodylate buffer containing 0.2 M sucrose, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at 4 °C for 1 h, dehydratated and embedded in DER. Thin sections were double-stained with uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope.

Study of the ingestion process and the associated metabolic stimulation in CHS and control cells. To evaluate the ingestion process in CHS and control leukocytes, 1 ml of whole blood was incubated at 37 °C with heat killed *Staphylococcus aureus* (bacteria-cell ratio 20:1). After 5 min May-Grünwald- and Giemsa-stained smears were prepared, and the ingested bacteria were counted. $^{14}CO_2$ production from 1-^{14}C-glucose by resting and phagocytizing cells was measured as previously described [17].

Exocytosis. To measure the enzyme release from the cells, leukocytes (5–10 × 10^6/ml) were incubated in plastic tubes in the presence of the indicated stimulants of exocytosis. Following various periods of incubation at 37 °C the reaction mixtures were centrifuged at 300 *g* for 5 min at 4 °C. The cell-free supernatants were carefully separated from the pellets, which were resuspended to the original volume, with KRP or KRT. MPO activity was assayed [16] in the cells disrupted by sonication (Brandson Sonifier 3A, 10 sec). The percentage of the total cell MPO activity which remained cell associated after phagocytosis of latex beads or treatment with the ionophore A23187 and calcium ions, was taken as an indirect measure of extracellular release of enzyme. This approach was followed since the activity of exocytosed MPO undergoes a marked inhibition in the extracellular medium [8, 23]. Unspecific leakage of cytosol proteins from the leukocytes was monitored by assaying the activity of cytoplasmic lactate dehydrogenase (LDH) [3].

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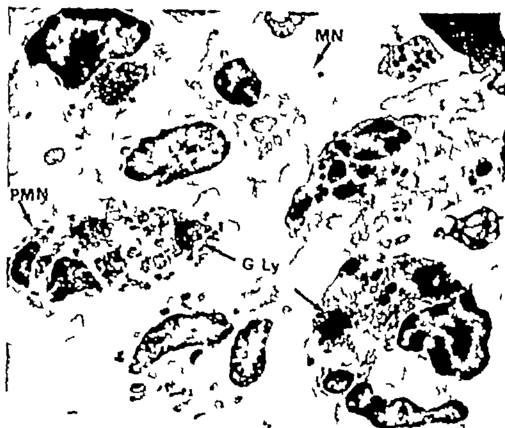


Fig 1 Electron-microscopic appearance of CHS neutrophils (PMN) and monocytes (MN) G Ly = Giant lysosome 8,400

cell damage Both control and CHS resting cells do not extrude either MPO or LDH

MPO release from leukocytes induced by ionophore A23187 and Ca² Normal cells after 15 min of incubation with 1 mM Ca² and 20 µM ionophore release about 60% of the total MPO activity into the incubation medium Conversely ionophore treated CHS leukocytes, as phagocytizing cells, extrude much less MPO (18%) and in any case this extrusion is paralleled by a leakage of LDH

Treatment of isolated granules with Triton X 100 To perform these experiments we have used the whole population of granules, which contain both the giant and the normal-size granules The free activity of MPO of granules from normal and CHS cells is virtually the same, i e 35 and 29% of total MPO activity respectively Furthermore by expos

Table 1 Release of MPO and LDH from phagocytizing cells (percent of the total cell-associated activity)

Incubation time, min	Control MPO	Control LDH	CHS MPO	CHS LDH
0	100	100	100	100
10	52	100	100	100
30	30	73	50	80

Averages of 2 experiments

ing the isolated granules to 0 005% Triton X 100 4 times as much MPO is solubilized from granules of normal leukocytes than from those of the CHS cells, i. e. 10 0 and 2.5 % of total MPO activity respectively

Discussion

Exocytosis from granulocytes is a multi-step process, which is very likely modulated by the cytoplasmic concentration of Ca^{++} [19 23] and involves the activation of contractile systems [1 21 24], the translocation of granules towards the invaginated plasma membrane and the fusion between the membrane-limited granules and vacuoles with discharge of the granular content into the vacuole Evidence that phagocytizing CHS leukocytes discharge the granule-associated enzymes in an abnormal fashion is widely accepted [11 18, 20] The defect may be ascribed to an alteration of any step from the generation of the exocytotic stimulus to the actual extrusion of enzymes into the phagocytic vacuole. For example, WHITE [22] has suggested that the alteration is localized at the level of the lysosomal membrane, while OLIVER *et al.* [9] and OLIVER and ZURIER [10] suggest that these cells have functionally altered microtubules.

From the results presented in this paper it emerges that the defective exocytosis of CHS leukocytes does not likely depend on an impaired mobilization of Ca^{++} from intracellular stores. In fact, by artificially increasing the cytoplasmic Ca^{++} concentration by means of the ionophore A23187 no enhancement in extrusion of granule-associated enzymes from the cell is observed. Our results point to an alteration of the properties of the lysosomal membrane In fact, the detergent Triton X 100 caus-

es a much lower solubilization of MPO from CHS granules than from normal leukocytes. This alteration might consist in an abnormal lipid-protein ratio and/or lipid composition of the membrane, which according to the more recent views on the mechanism of membrane fusion [2] would impair or slow down both the fusion between granules and phagocytic vacuoles, and the interaction with other cytoplasmic structures such as microtubules.

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Sea Blue Histiocyte Syndrome in Thai Siblings

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Key Words. Histiocytosis Liver cirrhosis Sea-blue histiocyte syndrome

Abstract We report two cases of sea blue histiocyte syndrome in Thai siblings. The abnormal histiocytes were found in the bone marrow and liver of both patients, but none was found in the other members of the family Cirrhosis and absence of axillary hair were present in both patients. One patient also had elephantiasis of the legs and Klinefelter s syndrome. Our studies support an autosomal recessive inheritance.

MOESCHLIN [5] in 1947 first described histiocytes that contained packed blue granules in the spleen of a patient with asymptomatic splenomegaly and WEWALKA [14] in 1950 found similar cells in the bone marrow SAWITSKY *et al* [8] in 1954 described two adults with hepatosplenomegaly and the bone marrow was loaded with similar cells. Subsequently SILVERSTEIN *et al* coined the term sea-blue histiocyte (SBH) syndrome to characterize these patients [11 13] Recently review of patients with the syndrome and criteria for diagnosis have been described [9 12] In several instances, more than one member of the same family were found to be affected [1 3 17] Recently we encountered two siblings who had clinical and histologic manifestations consistent with this entity Since this syndrome has not been previously described in this part of the world in addition there were some unique findings in our patients, we feel that the following report is worthwhile

Case Reports

Patient 1 On February 17 1972, a 19 year-old male from the south-eastern province of Thailand was admitted to Siriraj Hospital because of recurrent ulcers of

both legs. Two years before, multiple p cvs nod a cu leg They subsequently ulcerated and forme cers A p w th t ed for the leg ulcers. Abdominal mas a been h en child. Physical examination revealed sm nd pal g h o eight only 140 cm. The eyelids were puffy and e epica l ic d Bot axillary and pubic hair were absent. The p ns and ta norr m d soft the voice, however was hoarse. Both carrying angles and t ere as posterior dislocation f the right elbow T er d ea h lt fi finbers' breadth below costal margins Many scar w gh leg. The left leg which was swollen contained many nod b varying size een 0 5 and 3 cm in diameter Some f these nodules were ed and vered ith serous discharge. Hemoglobin 9.96 g*/a, hematocrit 3.15* W cells (WBC 3,850 µl with 40-56* of eosinophils on several determinations The blood cells were slightly hypochromic with moderate anisopoikilocytosis. The h bin electrophoresis was normal *Ascaris lumbricoides* and hook orm ova ent in the stool Biochemical values including bilirubin, SGOT SGPT and acid phosphatase were within normal limits Albumin 2.7 g*/s, globulin 6 1 g* The culture of fluid from the ulcerated nodules of the left leg yielded *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Lymphangiography of the left leg revealed deep lymphatic obstruction. Thick films for Microfilaria performed twice during the nighttime were negative The biopsy of the left inguinal lymph node revealed non-caseous granuloma with reactive hyperplasia. The testicular biopsy revealed trophic seminiferous tubules with hyperplasia of interstitial cells. The buccal smear demonstrated Barr bodies, and karyotype study was XXy The 24-hour values f 17-ketosteroids and 17-hydroxycorticosteroids were normal, whereas the leve of follicle-stimulating hor mone (FSH) was 102 mouse units (MU normal 6-52 MU) The bone marrow aspiration revealed increased cellularity prominent eosinophils and plasma cells. The number of megakaryocytes was normal, but many showed peripheral vacuolization Numerous large cells with typical sea-blue inclusions were seen (fig. 1).

Patient 2. A 27-year-old female was admitted to Siriraj Hospital on November 7 1972, fter the diagnosis of SBH syndrome on her brother had been established. Since childhood, several episodes of asymptomatic jaundice of unknown duration had occurred, the last one was 1 year previously A slowly growing mass has lso been noticed t the left side of the abdomen since youth. Physical examination revealed moderate jaundice, with puffy eyelids and widened epicanthic folds. The xil lary and pubic hair were also absent The liver was not felt, and the spleen wa one hand' breadth below the costal margin. Hemoglobin 9.96 g* WBC 3,800/ l with 30-45*/ of eosinophils on several occasions. Total bilirubin 5 mg* with the direct fraction of 2.3 mg*/s, SGOT 76, SGPT 33, (normal 20-35), albumin 3.1 g* s, globulin 4 6 g* s. 24-hour urine for FSH showed 13 MU and for 17-ketosteroids 6.75 mg* The bone marrow showed slight increase in cellularity of all the blood element with abundant eosinophils. Numerous SBH were noted (fig 2)

Aspirated needle biopsy of the liver stained with HE and Masson trichrome stain of these two patients were quite similar They demonstrated distorted architecture due t the increase of connecti tissue around the portal areas and the area adjacent to large foam cells. These cells contained many granules hich did not

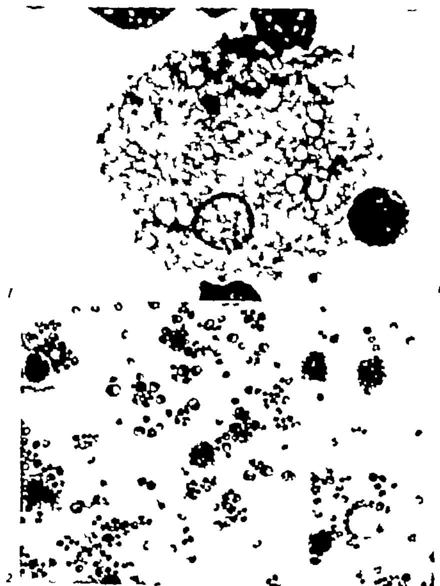


Fig 1 Bone marrow of patient 1 large histiocyte with sea blue granules in the cytoplasm Wright stain × 1 000.

Fig 2 Bone marrow of patient 2 numerous sea blue histiocytes. Wright stain, × 100

pick up the stain (fig 3 4) The granules of these cells, however stained positively with PAS and negatively with toluidine blue and Feulgen stain.

Our patients were the fifth and the last offsprings of their mother second marriage. The next elder brother of the propositus had died of progressive jaundice and coma at the age of 6. There was no history of consanguinity Almost all the mem-



Fig 3 Needle biopsy of the liver in patient 1 showing fibrocollagenous material densely accumulated near the vacuolated histiocytes. Masson stain, 250

Fig 4 Abnormal histiocytes in the liver of patient (arrow). Masson stain, 400.

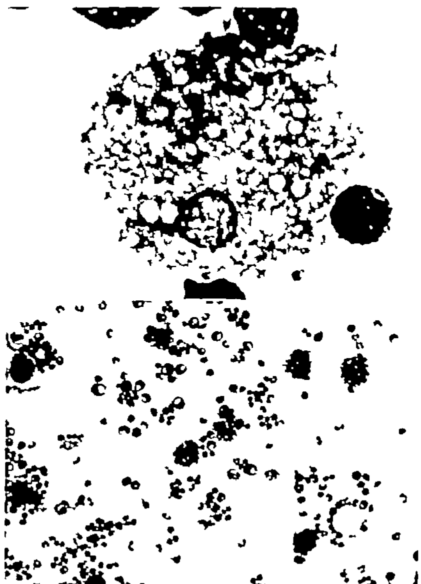


Fig 1 Bone marrow of patient 1 large histiocyte with sea-blue granules in the cytoplasm. Wright stain × 1 000

Fig 2 Bone marrow of patient 2. numerous sea-blue histiocytes. Wright stain, × 100.

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Fig 4 Abnormal histiocytes in the liver of patient 2 (arrow). Masson stain, 400

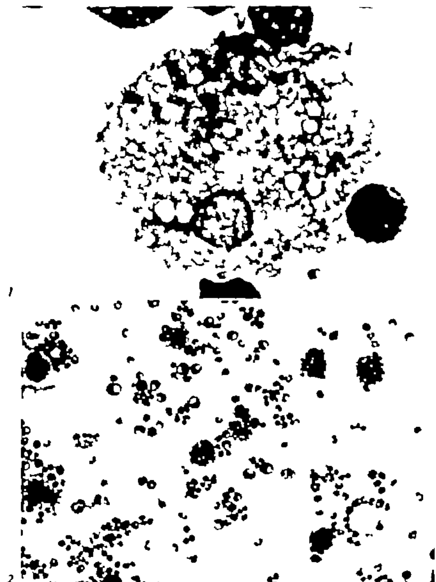


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that the obstruction of the lymphatic drainage might be caused by the aggregation of the abnormal histiocytes in the lymph node. The histology of the inguinal lymph node, however, did not substantiate this, and the presence of non-caseous granuloma with giant cells has been described by others [1, 14].

The presence of splenomegaly, SBH in the bone marrow and liver were only found in two members of the family. This supports an autosomal recessive inheritance for this syndrome as suggested by others [3, 9, 17]. Our patients probably are homozygous and, in accord with SAWITSKY *et al.* [9], we found no patient who could be described as heterozygous [17].

Diminution of the axillary and pubic hair has not been reported in SBH previously. They were found in both patients and also among several members of their family. Although in the first patient this can be explained as part of the manifestations of Klinefelter's syndrome, it failed to apply to the others. Hypopituitarism can be excluded in both patients regarding the normal values of urinary steroid metabolites and normal thyroid function. This unexplained abnormality could be genetically transmitted through the family and may be just purely coincidental. However, it might be wirthwhile in the future to look for hair abnormality in further cases of SMB syndrome.

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bers of the family did not give any history of mass in the abdomen, jaundice, or early death. Physical examination, biochemical studies including cholesterol, hemogram, liver function tests, and bone marrow examination were done on the patients parents, two brothers, and one sister (first marriage), and none had enlarged liver and spleen. The father however had a congenital deformity of the left hand with rudimentary fingers and thumbs. One of the sister and brother of the propositus also had scanty axillary and pubic hair Except for iron deficiency anemia in the mother and both patients, there was no SBH in the bone marrow or other hematologic abnormalities.

Comment

The clinical manifestations shown by the patients and the morphology of the abnormal cells were similar to SBH syndrome described previously [11] This condition differs from the juvenile form of Niemann Pick disease, Gaucher's disease, Hurler's disease and lipogranulosis. Lipid histiosis of the spleen occasionally observed in diabetes, Cooley's anemia and ITP can be excluded [2, 4 7] SCHIFF *et al* [10] recently have described hepatosplenomegaly in two asymptomatic siblings, and analysis of the vacuolated liver cells contained cholesterol ester Foamy histiocytes were not present in the bone marrow or in the liver Recently YAMAMOTO *et al* [15 16] reported a drug associated sea blue histiocytosis in which accumulation of acid phospholipids in the form of lysol-bis-phosphatidic acid was found in the tissue of their patient and their experimental animals. The inducing agent was 4-4-diethylamino-hexestrol which has been used clinically as a vasodilator Our patients did not have a history of such drug ingestion

Although hepatosplenomegaly is nearly universal only a few patients had cirrhosis [9] Our patients probably had cirrhosis due to the distorted architecture. It is noteworthy that the most intense fibrosis occurred in *the areas adjacent to the foamy histiocytes which were probably the same* kind of cells seen in the bone marrow This phenomenon might be the reaction of the liver cells to the injurious effect produced by the abnormal histiocytes, or possibly the SBH stimulates the formation of collagen The lack of inflammation and necrosis of the liver cells around the histiocytes seems to support the later view

The cause of elephantiasis in our first patient was not identified. *W bancrofti* was probably not the cause since it is a regional disease and has never been found in the area where our patients resided. The failure to recover the organism by proper search tends to support this. It was possible

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An *in vitro* Demonstration of the Ability of Human Bone Marrow Stromal Elements to Sustain Granulocytopoiesis

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Key Words. Marrow stromal elements · Granulocytopoiesis · Colony stimulating factor

Abstract. The ability of human bone marrow particles to produce a microenvironment conducive to granulocytopoiesis was tested by culturing them *in vitro* without an exogenous source of colony stimulating activity (CSA). Granulocytopoiesis in this system was confirmed by the following observations: (1) presence of mitotic figures in promyelocytes and myelocytes; (2) early disappearance of mature granulocytes, followed by their reemergence after 4 days in culture, and (3) presence of immature granulocytes even after 10–14 days in culture. Although no exogenous source of CSA was added to the culture plates, a probable endogenous source was the dense accumulation of stromal elements in the core of particles; these cells may generate sufficiently high local levels of CSA to stimulate and nurture granulocyte proliferation and maturation.

The classical *in vitro* model of granulocytopoiesis involves the production of colonies in a semisolid medium to which a suspension of bone marrow cells has been added [1–14]. This system appears to require two major elements: 1) committed granulocyte precursor cells which are capable of proliferating and maturing in the presence of an appropriate hormonal stimulus-colony stimulating activity (CSA), and, (2) a source of CSA usually exogenously provided by a feeder layer of peripheral blood [16], spleen cells [1], embryo kidney cells [14], or by the addition of serum, urine, or media conditioned by any of a variety of cell types [17]. When human bone marrow suspensions are studied, CSA may be generated endogenously if relatively high cell concentrations ($>5\times10^5$ cells/cm^2) are used. This autostimulatory phenomenon appears to be due to the

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Table I

Patient	Age	Diagnosis	Proliferating cells, %	Maturing cells, %
1	19	Hodgkin disease stage 2B (in remission)	49.8	50.2
2	16	Ewing sarcoma (marrow negative for tumor cells)	66.6	33.3
3	43	Felty's syndrome	80	20
4	75	polycythemia vera	47.8	52.8
5	18	osteogenic sarcoma (marrow negative for tumor cells)	60.2	39.8

myelocytes, bands, polymorphonuclear leukocytes) cells were then calculated. Some particles were also stained for α-naphthyl acetate esterase activity [4]

Results

Microscopic examination of marrow particles under low power (4× lens) initially showed a dense core interspersed with fat globules and a thin rim of cells at the periphery (fig. 1). Within 24 h this outer rim of cells had expanded and by 3–4 days of culture a thick outer layer of cells was apparent with many of the cells migrating individually into the surrounding milieu (fig. 2).

Microscopic examination of smears of marrow particles demonstrated that the tight inner core was comprised primarily of mononuclear cells the morphology and histochemical (α-naphthyl acetate esterase) reactions of some of these cells suggested they were histiocytes. Around this core were fat globules and hematopoietic cells of erythroid, myeloid, and megakaryocytic nature Although the relative proportions of proliferative (blasts, promyelocytes, myelocytes) and maturing (metamyelocytes, bands, polymorphonuclear leukocytes) cells were quite variable initially (table 1), they became quite uniform as the cultures matured *in vitro* (fig 3)

Within the first 24 h of culture, maturing neutrophils (beyond myelocyte stage) were in various stages of deterioration by contrast, promyelo-

presence of a sufficient number of monocytic cells in the suspension to generate levels of CSA adequate for granulocytopoiesis [11].

In vivo, however, bone marrow does not exist as a suspension of individual cells but rather as an organized tissue composed of two major elements: (1) hematopoietic cells which proliferate and mature in response to appropriate regulatory stimuli, and (2) stromal elements (fat cells, histiocytes, adventitial cells, sinus and endothelial cells, osteoblasts, osteoclasts, and fibroblasts) which provide the supporting framework and possibly some of the regulatory stimuli for hematopoiesis. Evidence for the regulatory role of stromal elements *in vivo* comes from numerous studies including those of MALONEY and PATT [8] and KNOSPE *et al.* [6]. Bone marrow particles, which contain developing myeloid cells held in close proximity to each other as well as to stromal elements by gelatinous material, may thus provide a closer *in vitro* approximation of *in vivo* granulocytopoiesis than do suspensions of individual bone marrow cells. In order to test this concept, the ability of human bone marrow particles to induce a microenvironment capable of initiating and sustaining granulocytopoiesis *in vitro* independent of exogenous stimuli was studied.

Methods

The culture technique: 1 cm³ of bone marrow from teenage and adult patients with a variety of disorders (table I) was aspirated into a plastic 3.0 ml syringe containing 0.05 ml of preservative-free heparin (5 000 U/ml – Gibco Diagnostics, Grand Island, N.Y.); the marrow was then transferred to a plastic test tube (Falcon Plastics, Oxnard, Calif.). After 1–3 min, marrow particles usually rose to the surface and were transferred by Pasteur pipette to 1 ml of modified McCoy's 5A medium (McCoy's 5A medium plus 15% fetal calf serum and added nutrients) [16]; the tube was shaken and the particles were again allowed to rise to the surface. They were removed by Pasteur pipette and transferred to a second test tube containing 1 ml modified McCoy's 5A medium. After this second wash the particles were removed by Pasteur pipette and added to a plastic test tube (Falcon) containing 10 ml of modified McCoy's 5A plus 1.6% methyl cellulose (Dow Methocel viscosity 4 000 cps) prepared according to the method of ISCOVE [5]. The tube was inverted several times to mix the particles with the culture medium and then 1 ml aliquots containing from 2 to 5 particles were cultured in 35 × 10 mm plastic Petri dishes (Falcon) at 37 °C in 10% CO₂ in air.

Particles were removed by Pasteur pipette on a daily basis, smeared on glass slides, and Wright stained for microscopic examination. At least 5 smears were prepared daily and differential counts of 200 cells per smear were obtained. The percentages of proliferating (blasts, promyelocytes, myelocytes) and maturing (meta-

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In vivo however bone marrow does not exist as a suspension of individual cells but rather as an organized tissue composed of two major elements (1) hematopoietic cells which proliferate and mature in response to appropriate regulatory stimuli, and (2) stromal elements (fat cells, histiocytes, adventitial cells sinus and endothelial cells, osteoblasts, osteoclasts, and fibroblasts) which provide the supporting framework and possibly some of the regulatory stimuli for hematopoiesis Evidence for the regulatory role of stromal elements *in vivo* comes from numerous studies including those of MALONEY and PATT [8] and KNOSPE *et al* [6] Bone marrow particles, which contain developing myeloid cells held in close proximity to each other as well as to stromal elements by gelatinous material may thus provide a closer *in vitro* approximation of *in vivo* granulocytopoiesis than do suspensions of individual bone marrow cells. In order to test this concept, the ability of human bone marrow particles to induce a microenvironment capable of initiating and sustaining granulocytopoiesis *in vitro* independent of exogenous stimuli was studied.

Methods

The culture technique 1 cm³ of bone marrow from teenage and adult patients with a variety of disorders (table I) was aspirated into a plastic 3.0 ml syringe containing 0.05 ml of preservative free heparin (5,000 U/ml – Gibco Diagnostics, Grand Island, N Y) the marrow was then transferred to a plastic test tube (Falcon Plastics, Oxnard, Calif.) After 1–3 min, marrow particles usually rose to the surface and were transferred by Pasteur pipette to 1 ml of modified McCoy's 5A medium (McCoy's 5A medium plus 15% fetal calf serum and added nutrients) [16] the tube was shaken and the particles were again allowed to rise to the surface. They were removed by Pasteur pipette and transferred to a second test tube containing 1 ml modified McCoy's 5A medium. After this second wash the particles were removed by Pasteur pipette and added to a plastic test tube (Falcon) containing 10 ml of modified McCoy's 5A plus 1 6% methyl cellulose (Dow Methocel viscosity 4,000 cps) prepared according to the method of ISCOVE [5] The tube was inverted several times to mix the particles with the culture medium and then 1-ml aliquots containing from 2 to 5 particles were cultured in 35 × 10 mm plastic Petri dishes (Falcon) at 37 °C in 10% CO in air

Particles were removed by Pasteur pipette on a daily basis, smeared on glass slides, and Wright stained for microscopic examination. At least 5 smears were prepared daily and differential counts of 200 cells per smear were obtained The percentages of proliferating (blasts, promyelocytes, myelocytes) and maturing (meta

Table I

Patient	Age	Diagnosis	Proliferating cells, %	Maturing cells, %
1	19	Hodgkin s disease stage ₋B (in remission)	49 8	50.2
2	16	Ewing's sarcoma (marrow negative for tumor cells)	66 6	33.3
3	43	Felty syndrome	80	20
4	75	polycythemia vera	47.8	52.8
5	18	osteogenic sarcoma (marrow negative for tumor cells)	60 2	39 8

myelocytes, bands, polymorphonuclear leukocytes) cells were then calculated. Some particles were also stained for α-naphthyl acetate esterase activity [4]

Results

Microscopic examination of marrow particles under low power (4× lens) initially showed a dense core interspersed with fat globules and a thin rim of cells at the periphery (fig. 1). Within 24 h this outer rim of cells had expanded, and by 3–4 days of culture a thick outer layer of cells was apparent with many of the cells migrating individually into the sur rounding milieu (fig. 2)

Microscopic examination of smears of marrow particles demonstrated that the tight inner core was comprised primarily of mononuclear cells the morphology and histochemical (α-naphthyl acetate esterase) reactions of some of these cells suggested they were histiocytes. Around this core were fat globules and hematopoietic cells of erythroid, myeloid, and megakaryocytic nature. Although the relative proportions of proliferative (blasts, promyelocytes, myelocytes) and maturing (metamyelocytes, bands, polymorphonuclear leukocytes) cells were quite variable initially (table 1), they became quite uniform as the cultures matured *in vitro* (fig. 3)

Within the first 24 h of culture, maturing neutrophils (beyond myelocyte stage) were in various stages of deterioration by contrast, promyelo-





Fig 1 Freshly aspirated bone marrow particle after washing and suspension in methylcellulose

Fig 2 Bone marrow particle after 4 days in culture.

cytes and myelocytes remained viable and were often observed to be in mitosis As shown in figure 4 proliferative cells accounted for 86% of the neutrophil series by day 2 and maintained this level for the next several days. On day 4 mature neutrophils began to reappear in significant numbers and equalled the proliferating elements by day 6–7 Mature elements became more predominant as the cultures aged until day 11–12 when the granulocytic cells began to deteriorate and disappear from the particles, which were then composed primarily of histiocytic cells However some promyelocytes and myelocytes remained in evidence throughout this period and were noted even as late as day 14 of culture

In addition to the granulocytopoiesis occurring around the particles,

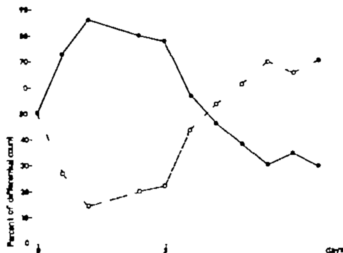


Fig 3 Graph depicting daily changes in the granulocytic population of the bone marrow particles. Proliferating cells refers to myeloblasts, promyelocytes, and myelocytes while maturing cells are metamyelocytes, bands, and polymorphonuclear leukocytes ● = Mitotic cells, O = maturing cells.

there was also proliferation and maturation of mononuclear cells. These cells varied in morphology from small forms with deeply basophilic cytoplasm and small condensed nuclei to large histiocytic and epithelioid-like cells. Many of these cells had α-naphthyl acetate esterase activity As the cultures aged these cells became more predominant until by day 14 they comprised almost the entire population.

Although the dense packing of cells within the particles made precise quantitation of the population of the cultures impossible, most particles were found to contain several hundred to several thousand granulocytes and mononuclear cells.

Discussion

Numerous studies of bone marrow cultured in nutrient media have been made since Carrel and Burrows [2] and Lewis and Lewis [7] Woodliff [19] has cultured bone marrow particles in agar in the presence of normal human serum and noted the following sequence of events.

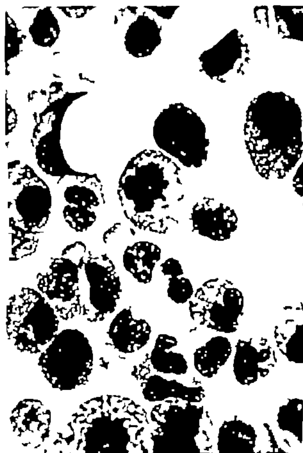


Fig. 4. Wright-stained smear of bone marrow particle after 7 days *in vitro*. Note maturing granulocytes at all stages from promyelocyte to polymorphonuclear leukocyte. Also present is a myelocyte in mitosis.

soon after incubation granulocytes migrated from the explant to form a halo around it other motile cells such as monocytes and lymphocytes also migrated out At 2 days the preparation contained degenerating cells as well as some healthy cells and a few mitotic figures. By 3 days most cells were degenerating or dying. Studies by OSGOOD [12] also demonstrated the presence of polymorphonuclear leukocytes *in vitro* for several days, but the majority had disappeared within the first 4 days.

The major differences between the study reported here and the results of previous studies are (1) the absence of human serum or other sources of CSA in the medium (2) persistence of promyelocytes up to the 14th day of culture and (3) increases in metamyelocytes, bands and polymor

phonuclear leukocytes beyond 120 h of culture with these cells reaching their maximum numbers at the 9th and 10th days of culture.

This study in addition to those cited above, may now be interpreted in the light of data on the requirement of CSA to stimulate and sustain granulocyte colony formation from suspensions of individual hematopoietic cells [9 10 18] Although no exogenous source of CSA was added to the marrow particle cultures in this study granulocyte proliferation and maturation was observed to occur Evidence that granulocytopoiesis had taken place may be summarized as follows (1) the presence of mitotic figures in promyelocytes and myelocytes throughout the period of culture up to and including day 12 *in vitro* (2) the deterioration of mature granulocytes in the first few days of culture followed by their reemergence after 3–4 days *in vitro* and their continued production up to the 14th day of culture, and (3) the presence of promyelocytes and myelocytes throughout the culture period up to and including day 14

It appears likely that the microenvironment created by stromal elements within the particles (most likely the dense core of histiocytic cells) was sufficient to initiate and sustain granulocyte proliferation and maturation. This may be the result of cell contact processes or the elaboration of high local concentrations of CSA and other regulatory factors. Such local milieux are apparently scattered throughout the human bone marrow

These results, which suggest a specific role for bone marrow stroma in initiating and sustaining human granulocytopoiesis, are consistent with those of Chan and Metcalf [3] using a murine system. In their studies, cells adherent to the bone shaft after hematopoietic cells had been flushed from the marrow cavity were able to generate 8–20 times as much CSA as were the extruded marrow hematopoietic cells. Their conclusion that local production of CSA in the mouse bone marrow is dependent upon cells concentrated at the periphery of the marrow cavity and/or adherent to the bony wall of the cavity is consistent with the fact that mouse marrow hematopoietic cell suspensions have an absolute requirement for exogenous sources of CSA in order for granulocytopoiesis to occur *in vitro* The situation for human bone marrow is somewhat different in that granulocyte colony production can occur *in vitro* without an exogenous source of CSA *provided that a high enough cell concentration [16] or intact marrow particles are used.* This autostimulation phenomenon suggests that CSA-producing cells are more uniformly distributed throughout the human bone marrow than they are in the murine system.

Regardless of the distribution of the cells which produce CSA, its local

concentration within the marrow cavity must be critical in regulating granulocyte production for it is in the marrow that the responsive precursor cells are located. Consequently generation of a given quantity of CSA or other regulatory factors locally by stromal elements within the marrow will be much more effective than the same quantity produced at diverse sites outside this organ Steady state granulocyte production could thus be regulated by the response of these stromal elements to systemic stimuli such as general tissue injury [10] or intermittant endotoxemia [15] In situations involving localized damage to peripheral tissues (e.g. cellulitis, pneumonitis, pyelonephritis) CSA produced by local histiocytes could increase the baseline marrow concentration of this regulator and thus lead to augmented granulocytopoiesis.

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Regulation of Human Hemopoietic Stem Cell Proliferation by Syngeneic Thymus-Derived Lymphocytes

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Key Words. Hemopoiesis · Lymphocytes · Stem cells

Abstract. Viable T lymphocytes stimulate the proliferation of human syngeneic hemopoietic stem cells, but not influence their differentiation. The biological significance of this activity is discussed and its possible physiological role in the regulation of hemopoiesis is considered.

Recently, we reported a definitive physical and immunological characterization of the human hemopoietic stem cell (HSC), and its successful growth in culture [3]. Subsequently, we have been concerned with studying possible mechanisms for regulating the proliferation and differentiation of this cell *in vivo*, and evaluating their physiological relevance.

Leukocytes have long been known to stimulate the growth of other cells *in vitro*, but it is only relatively recently that a lymphocyte mitogenic factor was described [14]. The immunological importance of such endogenous mitogens, in human lymphocyte populations, is well established.

Pluripotent HSC give rise to progenitor cells committed to a single pathway of differentiation. The unipotent progenitor of granulocytes and macrophages can be readily cultured *in vitro* (CFU-C), and by this means such cells have been clearly demonstrated not only in human bone marrow but also in peripheral blood. Stimulation of the growth of CFU-C by allogeneic lymphocytes has been reported in studies on murine [15, 18] and human bone marrow [7, 20].

In vivo growth of true HSC in mice has been widely studied with the

[1] We wish to thank ELAINE LEE, ADA BROOKS and TURID KNUTSEN for expert technical assistance.

spleen colony assay. By this technique, it has been shown, that, in the mouse, the proliferation of HSC can be stimulated by thymus-derived lymphocytes (T cells) [1, 4, 6, 13]. Unfortunately this method is inapplicable to man. However, when hemopoietic tissue is grown in Millipore diffusion chambers, implanted intraperitoneally in lethally irradiated mice, the proliferation and differentiation of HSC are supported [3]. Such *in vivo* cultures are successful even when xenogeneic cells are used. The sustained growth of human hemopoietic tissue, in this system, may be due to the production of soluble mitogens [16] or other factors produced by endogenous lymphocytes.

Our present study reports an assessment of the influence of syngeneic T lymphocytes on the *in vivo* growth of human HSC.

Materials and Methods

Peripheral venous blood was harvested from normal adult volunteers, and stem cell concentrates and pure T- and B-cell suspensions were prepared as previously described [5]. The HSC concentrates and pure B-cell suspensions were cultured in Millipore diffusion chambers, alone (X) and in 1:1 combination with pure, syngeneic T cells (Y) or T cells irradiated to 2,500 R (Z), the total number of cells implanted (2×10^6 per chamber) being the same in all cases. With appropriate transplantation to additional lethally irradiated hosts, two chambers were harvested from each of the six groups, on each of 4 consecutive weeks. Total and differential cell counts were performed on the contents of each chamber. Technical details relating to implantation, culture and harvest are given by BENESTAD [5].

Results

The results of one harvest from such an experiment are given in table I. Comparison of X with Y yields information on the effect of viable T cells on the differentiation of syngeneic B cells and HSC under *in vivo* culture conditions. It may be seen that T cells exert no influence on differentiation of HSC; in particular, the proportional representation of the major cells lines (granulopoietic, erythropoietic and megakaryopoietic) in the HSC cultures is not significantly altered (evaluated in four complete experiments).

Manifestation of mitogenic activity by the lymphocyte is dependent on its retention of an intact mechanism for RNA synthesis [21]. Hence, comparison of Y with Z affords an estimation of the ability of viable T-cells

Table I Harvest of 20 day cultures from intraperitoneal Millipore diffusion chambers

Mean cell counts	T lymphocytes W	Stem cell concentrate X	Y	Z	B lymphocytes X	Y	Z
Total $\times 10^{-5}$	0.62	2.28	19.52	1.83	0.64	4 17	0.46
Differential %							
Lymphocytes	100	52	82.5	68	62	98.5	82
Macrophages	–	8	3	1.5	38	1 5	18
Granulocytes	–	36	12	28.5	–	–	–
Normoblasts	–	3.5	2	1.5	–	–	–
Megakaryocytes	–	0 5	0.5	0.5	–	–	–

Cells implanted in duplicate chambers W = 2×10^5 T lymphocytes X = 2×10^4 cells from stem cell concentrate (SCC) or B lymphocytes Y = 1×10^5 SCC or B lymphocytes + 1×10^5 syngeneic T lymphocytes Z = 1×10^5 SCC or B lymphocytes + 1×10^5 irradiated syngeneic T lymphocytes.

to stimulate proliferation of syngeneic B-lymphocytes and HSC. The intimate inter relationship of T and B-cells is widely recognized so the striking increment in lymphocyte harvests ($p<0\ 001$) from the B-cell cultures was predictable In addition however viable syngeneic T cells produced a significant increment ($p<0\ 05$) in the recovery of the total progeny of HSC from cultures.

Discussion

These data indicate that the intact, viable T cell is capable of stimulating the proliferation of syngeneic HSC, but that it exerts no influence on differentiation Since peripheral blood T cells recirculate to bone marrow [11] it is conceivable that they may exert some form of physiological regulation on the proliferation of HSC. In the steady state HSC are generally believed to be dormant with respect to the proliferative cycle, being in a G_0 phase, and hence require to be triggered into proliferation It seems unlikely that the T cell would influence this phenomenon by emperipolesis [12] or even by trephocytic activity [10] While intercellular contact may be important [22] the available evidence suggests that mitogenesis is mediated by a soluble cell product

The reported heterogeneity of soluble mediators of mitogenesis pro-

duced by human leukocytes [19] should not inhibit the search for biological and biochemical similarities in these various activities, for the differences may be more apparent than real. Thus, when bone marrow is cultured in semi-solid agar media, the product is largely restricted to the progeny of CFU-C granulocytes and macrophages. Reduction in the amount of agar allows the proliferation but not the differentiation of HSC [9] In the Millipore diffusion chamber culture system, which is agar-free, HSC proliferate and differentiate, as previously noted, but when the chambers also contain agar only the growth of granulocytes and macrophages is supported [8] Hence, the presence of agar appears to favor CFU-C at the expense of HSC growth. Therefore, an assessment of the hemopoietic target cell for soluble mitogens of lymphocytic origin must take into consideration the nature of the culture system used. It may well be that lymphocytes produce a single mitogenic factor capable of acting on a wide variety of target cells. Further support for this hypothesis may be derived from the recent reports of the stimulatory effect of human leukocyte-conditioned medium on allogeneic CFU-E [2] and CFU-M [17] (the unipotent progenitors of erythrocytes and megakaryocytes, respectively)

This subject seems worthy of further study for the ever increasing number of operational definitions may be needed more to explain methodological inconsistencies than true physiological variations.

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Megakaryocytes	–	0.5	0.5	0.5	–	–	–

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The reported heterogeneity of soluble mediators of mitogenesis pro-

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Sensitization of Stabilized Fibrin to Urea Dispersion by Undiluted Plasma and Serum

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Key Words. Blood coagulation · Clot dispersion · Factor XIII · Fibrinogen · Fibrin stabilization · Fibrin urea dispersion

Abstract. Clots obtained from normal native platelet-poor plasma are dispersed by the addition of 5 M urea. Clots from plasma diluted >1/4 and clots from undiluted plasma, thoroughly washed, are not dispersed by urea, but are rendered susceptible to its dispersing action by prior incubation in normal undiluted plasma or serum. The same phenomenon was observed by incubating the otherwise insoluble clots in normal plasma or serum fractions precipitated at 33% saturation with $(NH_4)_2SO_4$. The serum fraction does not interfere with the incorporation of putrescine into casein, although it conditions the urea-dispersing action on stable clots.

Clot dispersion by 5 M urea is one of the tests currently used for evaluating plasma factor XIII (fibrin-stabilizing factor): cross-linked fibrin, contrarily to noncross-linked, is resistent to urea dispersion.

We have observed that the clot obtained from human native normal platelet-poor plasma is dispersed by urea [1] at a final concentration of 2.5 M. The following procedural details are essential to demonstrate this phenomenon: (1) Urea dispersion is evident only on a low range of plasma dilutions (up to 1:4): in fact an undispersible clot is obtained by increasing the dilution of plasma. (2) Dispersion time is variable (range 48–72 h): therefore incubation at 37 °C must be prolonged beyond this time. (3) *Instead of transferring the clot to the urea solution, the latter should* be added to the clotted sample (5 M urea is added in equal volume: final concentration of urea is therefore 2.5 M). This suggests the relevance of

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Table I. Effect of preincubation of stable clots in various media on urea clot dispersion

Clot type	Incubation media	Stability
Undiluted plasma clot (control 1)	–	0
Diluted plasma clot 1/8 (control 2)	–	+
	veronal buffer	+
	plasma	0
Washed undiluted plasma clot	33% plasma fraction	0
	45% plasma fraction	+
	60% plasma fraction	+
	serum	0
Washed diluted plasma clot	33% serum fraction	0
	plasma	+
	serum	+
	33% serum fraction	+

0 = Complete clot dispersion + = undispersable clot.
5 M urea is substituted with veronal buffer pH 7.4.

samples were tested: undiluted plasma, plasma diluted range 1/2 to 1/100 in Tris buffer pH 7.5, 0.3 M, and plasma diluted 1/4 in an aliquot of the same 33% serum fraction used in the clot dispersion test. Casein and plasma blanks were also tested.

Results

The results of the urea dispersion tests are summarized in tables I and II. Normal undiluted plasma clot (control 1) is dispersed by urea at a final concentration of 2.5 M (also in the presence of tranexamic acid), whereas the diluted plasma clot (control 2) is not. The washed undiluted and diluted plasma clots, preincubated in veronal buffer are undispersible· these undispersible clots, preincubated in undiluted plasma, serum and in the 33% fractions, are urea dispersible. The 45 and 60% plasma fractions are inactive.

The 33% serum fraction active in the urea dispersion test does not interfere with the incorporation of ^{14}C-putrescine into casein. In fact, normal plasma diluted 1/4 either in buffer or 33% serum fraction yield comparable cpm values (fig. 1).

plasma and serum to the dispersion phenomenon In this paper we report the results of experiments carried out to investigate their role in urea clot dispersion

Materials and Methods

Human platelet poor plasma and serum were obtained from normal donors. Serum was also obtained from the following animals. sheep, goat, horse, rabbit, guinea pig, cow rhesus monkey and Cercopithecus Clot dispersion by freshly prepared 5 M urea, unbuffered or buffered at pH 7 4 was carried out according to a technique described previously [1–3] with appropriate modifications. Plasma was collected using Na citrate 3.8‰ (1 part of anticoagulant to 9 parts of blood) this degree of plasma dilution is insignificant in relation to clot dispersion the clot will be referred to as undiluted plasma clot. Serum was obtained by centrifugation from *whole blood clotted spontaneously and left at room temperature for 6 h.*

Human clots were prepared by adding 0.1 ml of M/4 CaCl and 0.05 ml of thrombin (Roche 100 U/ml) to 1 ml of undiluted plasma and plasma diluted 1/8 with veronal buffer pH 7 4 0.8 M. 0.1 ml of Warner-Chilcott fibrinogen (12 mg/ml), heated at 40 C for 3 h, was added to the diluted plasma before clotting. After a 30-min incubation at 37 C, the clots were blotted on filter paper and washed three times in veronal buffer pH 7 4

Human plasma fractions were prepared by salt fractionation with $(NH_4)_2SO$ at final concentrations of 33 45 (range 33–45) and 60‰ (range 45–60) The precipitate was dissolved in saline (1/10 of the original plasma volume) and *dialyzed* against saline at 4 C for 72 h The 33‰ human serum fraction was prepared by the same procedure

The clots, prior to the addition of 1 ml of 5 M urea, were incubated for 1 h at 37 C in 1 ml of the following media veronal buffer pH 7 4 undiluted plasma and serum, and $(NH\ )_2SO_4$ fractions (table I)

Controls were prepared by adding 5 M urea in equal amount to plasma undiluted (control 1) or diluted 1/8 (control 2), after clotting washed undiluted and diluted plasma clots were also incubated in undiluted plasma, serum and 33‰ serum fraction substituting urea with buffer Control 1 was also prepared from plasma collected with tranexamic acid (10 mg/ml of plasma) to rule out the influence of fibrinolysis.

Dispersion of the undiluted plasma clot was also tested by adding different amounts of urea solution at different concentrations (table II). The animal serum was tested as follows. 0.1 ml Warner Chilcott fibrinogen, 0.1 of CaCl M/4 and 0.05 ml of thrombin were added to 1 ml of undiluted serum After a 30-min incubation at 37 °C, 1.25 ml of 5 M urea were added All the samples were incubated at 37 °C for 120 h and visually inspected at 6- to 12-hour intervals.

The influence of the 33‰ serum fraction on ^{14}C-putrescine incorporation into casein was investigated according to the method of DVILANSKY *et al* [4] ^{14}C-putrescine was purchased from the Radiochemical Centre, Amersham England. ^{14}C putrescine final concentration in the reaction mixture was 0.15 mM The following

Table I. Effect of preincubation of stable clots in various media on urea clot dispersion

Clot type	Incubation media	Stability
Undiluted plasma clot (control 1)	–	0
Diluted plasma clot 1/8 (control 2)	–	+
	veronal buffer	+
	plasma	0
Washed undiluted plasma clot	33 % plasma fraction	0
	45 % plasma fraction	+
	60 % plasma fraction	+
	serum	0
Washed diluted plasma clot	33 % serum fraction	0
	plasma	+
	serum	+
	33 % serum fraction	+

0 = Complete clot dispersion + = undispersible clot.
5 M urea is substituted with veronal buffer pH 7.4.

samples were tested: undiluted plasma, plasma diluted range 1/2 to 1/100 in Tris buffer pH 7.5, 0.3 M, and plasma diluted 1/4 in an aliquot of the same 33°/ serum fraction used in the clot dispersion test. Casein and plasma blanks were also tested.

Results

The results of the urea dispersion tests are summarized in tables I and II. Normal undiluted plasma clot (control 1) is dispersed by urea at a final concentration of 2.5 M (also in the presence of tranexamic acid), whereas the diluted plasma clot (control 2) is not. The washed undiluted and diluted plasma clots, preincubated in veronal buffer are undispersible these undispersible clots, preincubated in undiluted plasma, serum and in the 33°/₀ fractions, are urea dispersible. The 45 and 60°/ plasma fractions are inactive.

The 33°/ serum fraction active in the urea dispersion test does not interfere with the incorporation of ⁴C-putrescine into casein. In fact, normal plasma diluted 1/4 either in buffer or 33 / serum fraction yield comparable cpm values (fig. 1).

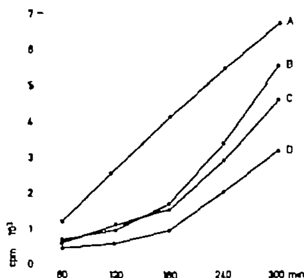


Fig. 1 ^{14}C-putrescine incorporation into casein in presence of 33% $(NH_4)_2SO_4$ human serum fraction. A = 33% serum fraction. B = undiluted plasma, C = plasma + serum fraction (1/4). D = plasma + buffer (1/4)

Table II Human undiluted plasma clot dispersion by urea: amounts of urea solution added to 1 ml of plasma and urea concentration are varied

Urea concentration %	amount ml	Clot stability
30	1	0
20	4	0
50	1	+
30	4	+

0 = *Complete clot dispersion* + = undispersible clot.

Among the animals tested, clot urea dispersion in the presence of undiluted serum is demonstrable only in the rhesus monkey and in the Cercopithecus.

Discussion

In previous publications [1–3] we have reported that the clot obtained from plasma undiluted or diluted 1/2 is dispersed by 5 M urea (30%)

provided it is added in equal amounts after clotting. An undispersible clot is obtained by increasing the dilution of plasma (>1/4) We thought that these data might be explained by the presence in normal plasma and serum of a factor XIII inhibitory activity which could be counteracted by dilution. Another possibility is that dispersion of the undiluted plasma clot might be the result of an activity that slowly sensitizes the stabilized clot to urea.

Incubation of washed undiluted and diluted plasma clot in undiluted plasma and serum, prior to addition of urea, indicates that plasma and serum are able to sensitize clots, already stabilized, to the dispersing action of urea this activity is recovered in the 33 / $(NH_4)_2SO$ plasma and serum fractions.

Dispersion of a stabilized clot implies a mechanism of action unrelated to factor XIII. To investigate this point the 33% $(NH_4)_2SO$ serum fraction was tested in the C-putrescine factor XIII assay The curves relative to the plasma dilutions 1/4 with buffer and serum fraction indicate that the same serum fraction that sensitizes a stabilized clot to urea dispersion does not interfere with the incorporation of putrescine into casein. This activity is not limited to humans but can also be demonstrated in the rhesus monkey and in the Cercopithecus.

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Binding of Deoxyribonucleic Acid to the Surface of Human Platelets

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Key Words. DNA binding Platelet surface Platelets DNA

Abstract It was demonstrated that washed human platelets can bind minute amounts of C DNA on their surface during short term incubation. The binding was specific and firm in the described experimental conditions. Washed platelets bound also ^{14}C-DNA anti DNA antibody complexes, although to a lesser amount than ^{14}C-DNA alone. The possible significance of these findings is briefly discussed.

In recent years several studies have shown that various tissues and cells, e.g., lymphoid tissue, fibroblasts from human skin and Chinese hamster lung, as well as plant cells take up deoxyribonucleic acid (DNA) when cultured in suitable media [1 6, 8, 10] whereas other cells, such as peritoneal macrophages from guinea pigs, failed to show uptake of DNA [8] In contrast to these well-documented results on the cellular uptake of DNA data on its binding capacity to cell membranes are scarce.

The present study was undertaken to find out whether the platelet can bind DNA in short term incubation The platelet has no DNA in its membrane [2] but is capable of interacting with certain large molecules that possess a repetitive structure [7]

Materials and Methods

Blood was obtained from normal volunteers and from systemic lupus erythematosus (SLE) patients.

For platelet preparations, standard hematological procedures were employed [3] After separation from the platelet-rich plasma, the platelets were washed three times

with 10 ml phosphate-buffered saline (PBS) (0.06 *M* phosphate buffer pH 7.2 in saline), *containing* Na_2 *EDTA (2 mg/ml) and finally resuspended either in PBS, or in* acetate buffer 0.06 *M* pH 5.6 containing Mg^{++} (5×10^{-4} mol/ml) in experiments in which DNase (100 μg/incubation mixture) was used. Contamination with red cells lymphocytes was less than 3%/

ml aliquots of washed platelet (WP) suspensions, containing $0.75–2.40\times10^{8}$ /ml, were incubated at 37 °C for 45 min with 1×10^{-7} g ^{14}C DNA (unless mentioned). After incubation, unbound ^{14}C DNA was eliminated by re-ing with PBS until no radioactivity was detected in the supernate. The rs vortexed and transferred to scintillation vials to which 10 ml Insta-ivity was measured with a Packard Tricarb Scintillation in counts/5 min.

washed red cells were used at concentration of ts. Serum anti-DNA antibody was determined by

complexes (DADAC) were obtained by treating with a predetermined quantity of either normal SLE sera (with 100% anti-DNA antibodies). At ^{14}C-DNA was complexed in the case of the quantity of ^{14}C DNA was complexed with

do and all the laboratory manipulations plastic tubes. ^{14}C-DNA, from *Escheri-* -stranded (radiochemical purity 100%), from *E. Coli* or from calf thymus for "biochemical purpose" was btained from Packard.

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Binding of Deoxyribonucleic Acid to the Surface of Human Platelets

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Key Words. DNA binding · Platelet surface · Platelets · DNA

Abstract. It was demonstrated that washed human platelets can bind minute amounts of ^{14}C-DNA on their surface during short-term incubation. The binding was specific and firm in the described experimental conditions. Washed platelets bound also ^{14}C-DNA anti DNA antibody complexes, although to a lesser amount than ^{14}C-DNA alone. The possible significance of these findings is briefly discussed.

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0.5-ml aliquots of washed platelet (WP) suspensions, containing 0.75–2.40×10^8 platelets/ml, were incubated at 37 °C for 45 min with 1×10^{-7} g ¹⁴C-DNA (unless otherwise mentioned). After incubation, unbound ¹⁴C-DNA was eliminated by repeated washing with PBS until no radioactivity was detected in the supernate. The cell pellets were vortexed and transferred to scintillation vials to which 10 ml Instagel was added. Radioactivity was measured with Packard Tricarb Scintillation Counter and results expressed in counts/5 min.

PBS suspensions of saline-washed red cells were used at concentration of 5×10^7 cells in control experiments. Serum anti-DNA antibody was determined by the method of Pincus [9].

¹⁴C-DNA anti-DNA antibodies complexes (DADAC) were obtained by treating 1×10^{-7} g ¹⁴C-DNA for 1 h at 37 °C with predetermined quantity of either normal (with 10% anti-DNA antibodies) or SLE sera (with 100% anti-DNA antibodies). At the end of the incubation, 10% of the ¹⁴C DNA was complexed in the case of the normal sera, whereas almost the entire quantity of ¹⁴C DNA was complexed with the antibodies in the case of the SLE sera.

All reagents were of an analytical grade and all the laboratory manipulations were performed in siliconized glassware or plastic tubes. ¹⁴C-DNA, from *Escherichia Coli (average mol.wt. 2×10⁹), double stranded (radiochemical purity 100%)*, was from Amersham, England. Unlabelled DNA, from *E. Coli* or from calf thymus and DNase were from Sigma Chemicals. RNA for "biochemical purpose" was from Merck (FRG). Intagel scintillation solution was obtained from Packard.

Results

Evidence that ¹⁴C-DNA was bound to the surface of the WP membrane and not incorporated into the cell, was obtained in two types of experiments.

First, WP were incubated for 30 min in PBS containing unlabelled DNA in concentrations of 50–100 times that of the labelled DNA in the binding experiments. The platelets were then washed to remove any unbound DNA and subsequently incubated with ¹⁴C DNA. At the end of the experiments, less than 10% ¹⁴C DNA, was platelet bound (representing nonspecific binding: table I, a) in comparison with the control (WP incubated with ¹⁴C DNA alone: table I b), thus indicating prior saturation of the binding sites with the labelled DNA.

In the second type of experiment, WP labelled with ¹⁴C-DNA and then

Table I The binding of ^{14}C DNA to washed platelets and the effect of treatment with excess unlabeled DNA or with DNase

	Number of experiment	Platelet number per incubation tube	^{14}C DNA bound, counts × 10^3/5 min (mean ± SD)
a) Platelets praeincubated with 50–100 excess unlabeled DNA	5	0.80×10^8	0.22 ± 0.04
b) Control	5	0.80×10^8	2.96 ± 0.26
c) DN-ase treated ^{14}C DNA platelets	3	1.00×10^8	0.17 ± 0.03
d) Control	3	1.00×10^8	2.60 ± 0.32

Table II The effect of SLE serum on the binding of ^{14}C DNA to washed platelets

	Number of experiments	Platelet number per incubation tube	Counts × 10^3/5 min (mean ± SD)
a) ^{14}C DNA labeled platelets treated with SLE serum	3	1.00×10^8	2.90 ± 0.32
b) SLE serum treated platelets incubated with ^{14}C DNA	3	1.00×10^8	1.15 ± 0.22
c) Control	3	1.00×10^8	2.95 ± 0.30

washed free from any unbound label, were incubated for 30 min with Dnase. They were then repeatedly washed and the radioactivity of the cell pellet measured. As shown in table I c, the DNase-treated platelets had lost more than 90% of their label in comparison with the control (table I d).

To test the specificity of the binding sites for DNA, experiments similar to those with unlabelled DNA were performed with unlabelled RNA. Prior treatment with RNA did not affect the capacity of WP to bind ^{14}C DNA subsequently to the same extent as the control (no treatment with RNA). The strength of the ^{14}C DNA platelet membrane bond was assessed by incubating WP with ^{14}C DNA before (table II a) or after (table II b) treatment with SLE serum. The control was represented by WP incubated with ^{14}C DNA alone (table II c). The data in table II demonstrate that in our experimental conditions the ^{14}C DNA membrane bond

was strong, since once the ^{14}C DNA was bound, the SLE serum was unable to detach it from the platelet.

Quantitative binding studies, using $0.2–2.5\times10^{-}$ g ^{14}C DNA were performed on WP from normals and SLE patients. The results show that 5.0×10^{7} normal platelets reached a binding saturation plateau at 11.0×10^{-9} g ^{14}C DNA, indicating a maximal binding capacity of the order of 100 molecules/cell. Preliminary results indicate that WP from SLE patients had a lower ^{14}C-DNA total binding capacity than that from normals.

In experiments carried out to investigate whether WP could bind ^{14}C DNA anti-DNA antibody complexes, the cells were incubated either with PBS containing SLE serum and ^{14}C-DNA entirely complexed with antibodies, or with normal serum and only 10% of ^{14}C DNA complexed with antibodies (see 'Methods'). The results showed that WP were capable to bind the complexes, although to a lesser extent than ^{14}C-DNA alone.

The binding of ^{14}C-DNA to WP was temperature dependent, since at 10 °C it was 65–70% of that measured at 37 °C. Control experiments with red cells suspensions did not reveal any binding of ^{14}C-DNA.

Discussion

The results demonstrate that human WP can bind, in short-term incubation, mintue amounts of ^{14}C-DNA and to a lesser extent ^{14}C DNA anti-DNA antibody complexes. The binding of ^{14}C-DNA was firm and specific in the described experimental conditions, as shown by the inability of SLE sera (with high content of anti-DNA antibody) to detach it from the platelet and by the failure of an excess of RNA to block the subsequently binding of the ^{14}C-DNA. Circulating DNA anti-DNA antibody complexes were described in some patients with SLE [5]. Whether there exists a causal relationship between the above shown capacity of WP to bind these complexes and the thrombocytopenia or the platelet abnormalities reported in some cases with SLE [4, 11] remains to be elucidated. The effect of the binding of DNA and of DADAC on the aggregation pattern of the platelets is currently under investigation.

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Metoprolol and the Peripheral Platelet Count

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Key Words. Peripheral platelet count · Platelet size · Metoprolol · Splenic platelet pool

Abstract. An acute oral administration of 50 mg metoprolol (a selective β-1 receptor antagonist) to 18 healthy volunteers induced a significant increase in the peripheral platelet concentration lasting more than 4 h. It is suggested that this increment in the platelet count originates from the exchangeable splenic platelet pool. The mechanism by which metoprolol exerts its effect remains to be established.

Introduction

Epinephrine given intravenously is known to cause an instant release of platelets from the exchangeable splenic platelet pool (ESPP) resulting in a concomitant increase in the peripheral platelet count [1, 2, 8]. An intravenous infusion of isoprenaline on the other hand causes a lowering of the platelet concentration and the results of recent studies carried out in our laboratory suggest that this effect is mediated via adrenergic β-1 receptors [9, 10]. These findings prompted us to investigate the effect of a selective β-1-receptor blocking agent (metoprolol) on the peripheral platelet count.

Material and Methods

18 healthy volunteers (9 males and 9 females) aged 18–49 years (mean 24) were selected for the present investigation. The subjects arrived at the laboratory at 8 a.m. After 10 min of rest in the recumbent position the heart rate was recorded, and using EDTA powder as anticoagulant venous blood was collected for the determination of platelet count, platelet size, percentage of megathrombocytes and haemato-

crit value. Thereafter the subjects received 50 mg of metoprolol tartrate (Seloken® Hässle, Sweden) orally and for the determination of the aforementioned parameters blood was collected at 90, 150, 210, 270 and 330 min. All blood sampling and heart rate recordings were made after 10 min of rest in the supine position.

The enumeration of platelets was performed by phase microscopy according to BRECHER *et al.* [4] The determination of platelet size was carried out as described previously [3] In brief, blood smears were made and the slides were stained with May-Grünwald-Giemsa. The platelet size (diameter) was determined with a calibrated ocular Zeiss micrometer and magnification of 1 200-fold. The diameters of 200 consecutive platelets were measured on the portion of the smear generally employed to determine red cell and white cell morphology Megathrombocytes were platelets with a diameter $>2.5\ \mu m$ [6] The haematocrit values were obtained by centrifugation of blood in capillary tubes at 13,460 g for 5 min (IEC MB micro-hematocrit centrifuge)

Standard statistical methods were employed. Unless otherwise stated mean values $\pm$ SEM are reported. Statistical analyses were made using the Student's t test and calculated on differences between matched pairs. The differences between means was considered significant if $p<0.05$

Results

The baseline mean heart rate was 65 ± 2 beats/min After 50 mg of metoprolol orally the heart rate decreased to a lowest mean value of 51 ± 2/min, and the bradycardia was maintained throughout the experiment.

The baseline mean platelet count (table I) was $224 \pm 16 \times 10^9$/l. In response to metoprolol there was a significant increase in the mean platelet

Table I The peripheral platelet count (No. $\times 10^9$/l) at different times after oral administration of 50 mg metoprolol to 18 healthy volunteers

	Baseline	Time				
		90 min	150 min	10 min	70 mm	330 min
Mean	224	237	245	24	35	230
SD	67	56	61	56	63	56
SE	16	13	14	13	15	13
t		2.8	4 8	3.8	2.5	1 2
p		<0.02	<0.001	<0.005	<0.05	n.s.

n.s. = Nonsignificant.

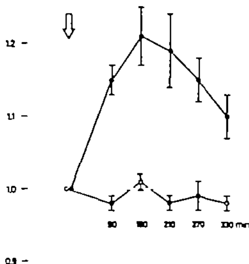


Fig 1 The platelet count (mean ± SE) at different times after metoprolol (●) and placebo (O) administration (arrow) to 5 healthy volunteers (see text).

count, and the highest mean value ($245 \pm 14 \times 10^9$/l) was obtained at 150 min. Thereafter the mean platelet count gradually decreased and the 330 min value was no longer significantly higher than the baseline value.

Five subjects were studied twice once using metoprolol and the sec ond time placebo The results of these experiments are shown in figure 1 The baseline platelet count was arbitrarily set as 1 0 and all subsequent values were related to the first value. It is seen that there was no change in the peripheral platelet count after placebo administration. Metoprolol on the other hand caused a considerable increase in the peripheral platelet concentration.

The mean baseline mean platelet diameter was 2.21 ± 0.21 μm (SD), and the mean percentage of megathrombocytes was 20 ± 10%/ (SD). These two parameters did not change during the experiment, nor was there any change in the haematocrit values.

Comment

The present study has shown that the peripheral platelet count is significantly elevated in response to acute oral administration of 50 mg of

metoprolol, a selective β-1 blocking agent. The maximum mean increase occurred at 150 min and amounted to around 9% over the baseline value. Thereafter the mean platelet count decreased and the 330 min value was no longer significantly elevated. This duration of effect is in accord with observations made by JOHNSSON *et al* [7] that after 50 mg of metoprolol the blockade of exercise-induced tachycardia is reduced by 50% within about 5 h.

Experimental work on animals has recently shown that an epinephrine injection in addition to thrombocytosis causes an increase in the percentage of circulating megathrombocytes [5] In the present experiments, however no change in the platelet size distribution occurred.

Although there is lack of direct evidence, it could be suggested that epinephrine releases platelets from the ESPP by α-receptor stimulation. The present study has demonstrated that the peripheral platelet count is increased in response to a selective β-1-blocking agent. In the absence of an alternate explanation we suggest that this increment in platelet count originates from the ESPP However the exact mechanism by which metoprolol affects the ESPP is unclear

Acknowledgements The authors wish to thank Miss MARIANNE DAHLBERG for her skilful technical assistance. This study was supported by Swedish Cancer Society (74.85) and Göteborg Medical Society

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Classic Hemophilia A in a Female

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Key Words Antihemophilic factor activity Antihemophilic factor antigen Hemophilia A Von Willebrand's disease Von Willebrand factor

Abstract A 53-year-old woman with a history of recurrent bleeding complications since childhood and a positive family bleeding history previously diagnosed as von Willebrand s disease, was investigated. She was found to have a markedly reduced level of antihemophilic factor (AHF) activity a low AHF activity/AHF antigen ratio, normal Ristocetin-induced platelet aggregation and a normal level of von Willebrand factor activity These findings were consistent with the diagnosis of classic hemophilia A which was confirmed by the results of similar studies in nine of the patient's relatives. The report illustrates the usefulness of newer laboratory methods in the differentiation between classic hemophilia A and von Willebrand's disease which may have important clinical implications.

Classic hemophilia A [antihemophilic factor (AHF)- factor VIII-deficiency] is an X-chromosomal disorder resulting in the synthesis of normal amounts of an immunoreactive AHF molecule (AHF antigen) with diminished or absent AHF-procoagulant activity [25] Few well-documented cases of clinically affected female hemophiliacs have been reported in the literature [13] In contrast von Willebrand s disease (vWD) is an autosomal, and probably heterogeneous, disorder [24] which occurs with equal frequency in males and females and is typically characterized by a reduction in the circulating blood of AHF activity as well as AHF antigen and a prolonged bleeding time due to the deficiency of a plasmatic factor (von Willebrand factor vWF) closely associated with the AHF molecule [17] However because of the known variability of the bleeding time in the same vWD patient and between different patients, vWD particularly in

mild form, cannot be excluded on the basis of a normal bleeding time. Thus, in the past, the demonstration in a female of low AHF activity (in the absence of an inhibitor) may have easily led to the diagnosis of vWD ("pseudohemophilia "vascular hemophilia,") even in the presence of a normal bleeding time. Over the last several years, test procedures have been developed which have greatly enhanced our ability to distinguish patients with classic hemophilia A and vWD This is not only of theoretical interest but may be of considerable importance in respect to the management of such patients during bleeding episodes or surgical procedures as well as genetic counseling. We report here the case of a 53-year-old woman with a moderately severe familial bleeding disorder previously diagnosed as vWD or "vascular hemophilia who was found to have classic hemophilia A by the use of these newer laboratory methods.

Case Report

This 53-year-old, single, white lady (R. M. L.) was referred for hemostatic evaluation prior to surgical removal of painful bunions on both feet. The patient gave history of easy bruisability since the age of 5, prolonged bleeding after minor skin lacerations, nosebleeds requiring packing on several occasions, and bleeding from gums with normal toothbrushing. She had experienced abnormally heavy and prolonged bleeding after tooth extractions in 1949 1952, and 1953, the last one requiring hospitalization for 7 days and several blood transfusions. In 1958, she had several episodes of bleeding from her rectum and bled excessively after subsequent hemorrhoidectomy requiring total of 60 blood transfusions over period of 6 weeks. At that time the diagnosis of mild AHF deficiency was first made on the basis of prolonged partial thromboplastin time which was corrected by adsorbed normal plasma. She had experienced heavy but not prolonged bleeding during menstrual periods which had been regular In 1967 she underwent operative removal of cervical polyp after which she bled intermittently for 3 months requiring several blood transfusions. Hemostatic evaluation at that time revealed prolonged partial thromboplastin time, "low" AHF activity mildly prolonged bleeding time, and positive Rumpel-Leede test which led to the diagnosis of "vascular hemophilia or von Willebrand's disease. This diagnosis was apparently confirmed on repeat laboratory examination at another hospital in 1970. A review of organ systems and physical examination at the time of admission to Barnes Hospital on October 30, 1974, revealed healthy appearing, moderately obese female with multiple bunions on both feet. Routine hematology and chemistry screening tests gave results within normal limits. The results of detailed hemostatic evaluation performed at that time and on subsequent visits since then are listed in table I, together with the results of

We thank Dr E. REINHARD for referral of this patient.

Table I Results of hemostatic evaluation of patient (R.M.L.) and nine relatives[1]

Hemostasis tests	Normal range	R.M.L. 1	A.L. 2	L.L. 3	L.N 4	J L. 5	S.L. 6	C.L. 7	R.L. 8	Li.L. 8	An.L. 10
Bleeding time, min	3–8	5.0	6.3	2.3	4.0	3.0	4.0	2.0	3.0	3.5	3.5
Platelet count ×1 000/mm³	150–400	260	350	445	285	300	250	280	255	270	320
PT sec	13.2–15.6	13.8	13.8	13.6	13.4	12.9	15.1	15.4	15.1	15.6	15.1
aPTT sec	20–38	43.1	26.8	25.3	25.8	40.8	26.8	30.8	29.0	29.5	29.4
Thrombin time, sec	12–18	13.9	12.3	12.9	13.1	13.6	14.9	14.6	13.3	14.8	16.3
Fibrinogen (Clauss), mg%	150–380	275	295	245	305	235	235	235	170	195	210
Clot urea solubility		neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
Whole blood clot retr		norm.	norm.	norm.	norm.	norm.	norm.	norm.	norm.	norm.	norm
Inhibitor (aPTT)		neg.	–	–	–	–	–	–	–	–	–
Platelet adhesiveness	>70%	80	81	87	70	95	–	–	–	–	–
Platelet aggregation											
ADP		norm.	–	norm.	norm.	norm.	–	–	–	–	–
EPI		norm.	–	norm.	norm.	norm.	–	–	–	–	–
COLL		norm.	–	norm.	norm.	norm.	–	–	–	–	–
RISTO		norm.	–	norm.	norm.	norm.	–	–	–	–	–
AHF activity U/ml	0.6–1.6	0.05	0.98	1.80	0.64	0.05	0.90	0.50	0.58	0.54	1.10
AHF antigen, U/ml	0.5–1.6	1.53	2.07	1.81	1.83	1.93	1.74	1.22	1.44	1.47	2.25
AHF act./ant. ratio	>0 61	0.03	0.47	0.99	0.34	0.03	0.52	0.41	0.40	0.37	0.49
vWF activity U/ml	0.6–1.5	1.10	1.38	0.92	1.12	1.58	1.30	1.06	0.92	1.34	1.30

[1] The figures below the initials of each individual tested refer to those shown in figure 1. The details of the test procedures are described in Materials and Methods. All tests (except platelet count and platelet function tests) were performed in duplicate and the mean values are shown.

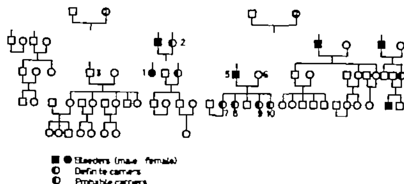


Fig. 1. Family pedigree of patient R. M. L. Those individuals identified as bleeders had experienced moderately severe or severe bleeding complications mostly following accidental injuries or dental or surgical procedures. Those identified as definite carriers had AHF activity/antigen ratios of <0.61. All individuals in whom laboratory studies were carried out are identified by numbers (table I).

similar studies performed on nine members of her family. The patient's pedigree is shown in figure 1. The patient's younger brother (J. L., No. 5), father and two uncles on her mother's side had abnormal bleeding manifestations, i.e., frequent nosebleeds, excessive bleeding after tooth extractions and large hematomas after injuries. A 5-year-old grandson of the patient's youngest uncle on her mother's side had easy bruisability and nosebleeds and bled excessively when he cut his lower lip during a fall.

The patient was discharged from Barnes Hospital on November 1, 1974, since, in view of her bleeding tendency, surgical intervention did not seem to be indicated. She underwent removal of her bunions at another hospital in June 1975. Postoperatively she developed icteric hepatitis from which she has not fully recovered.

Materials and Methods

Blood was collected from an antecubital vein (using a two-syringe technique) into 0.1 vol of 3.8% sodium citrate solution. Platelet-poor plasma (PPP) was prepared by centrifugation of the blood at 1,500 g for 10 min at room temperature. PPP was transferred with plastic pipettes into plastic tubes and stored in an ice bath for tests to be performed immediately or immediately frozen at -70 °C for tests to be performed later. Platelet-rich plasma (PRP) for platelet aggregation studies was prepared by centrifugation of citrated blood at 180 g for 10 min at room temperature. For the measurement of platelet adhesiveness, blood was collected into 10-ml plastic syringes containing 40 units of sodium heparin (The Upjohn Company, Kalamazoo, Mich.).

Table I Results of hemostatic evaluation of patient (R.M L.) and nine relatives[1]

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PT sec	13.2 15.6	13.8	13.8	13.6	13.4	12.9	15.1	15.4	15.1	15.6	15.1
aPTT sec	20–38	43.1	26.8	25.3	25.8	40.8	26.8	30.8	29.0	29.5	29.4
Thrombin time, sec	12 18	13.9	12.3	12.9	13.1	13.6	14.9	14.6	13.3	14.8	16.3
Fibrinogen (Clauss), mg %	150–380	275	295	245	305	235	235	235	170	195	210
Clot urea solubility		neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
Whole blood clot retr		norm.	norm.	norm.	norm.	norm.	norm.	norm.	norm.	norm.	norm
Inhibitor (aPTT)		neg.	–	–	–	–	–	–	–	–	–
Platelet adhesiveness	~70 %	80	81	87	70	95	–	–	–	–	–
Platelet aggregation											
ADP		norm.	–	norm.	norm.	norm.	–	–	–	–	–
EPI		norm.	–	norm.	norm.	norm.	–	–	–	–	–
COLL		norm.	–	norm.	norm.	norm.	–	–	–	–	–
RISTO		norm.	–	norm.	norm.	norm.	–	–	–	–	–
AHF activity U/ml	0.6–1.6	0.05	0.98	1.80	0.64	0.05	0.90	0.50	0.58	0.54	1.10
AHF antigen, U/ml	0.5–1.6	1.53	2.07	1.81	1.83	1.93	1.74	1.22	1.44	1.47	2.25
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[1] The figures below the initials of each individual tested *refer to those shown in figure 1* The details *of the test* procedures are described in Materials and Methods All tests (except platelet count and platelet function tests) were performed in duplicate and the mean values are shown.

patient had a normal level of AHF antigen with a low AHF activity/antigen ratio of 0 03 and a normal concentration of vWF in plasma. The results of similar studies carried out in nine of the patient's relatives, also shown in table I confirmed the diagnosis of classic hemophilia A in the patient's younger brother (J L., No 5) who was known to be a bleeder His four daughters (C.L., No 7 R.L., No 8 Li.L., No 9 An.L., No. 10) who had to be considered obligate carriers were all found to have abnormally low AHF activity/antigen ratios. Most importantly the patient's mother (A. L., No. 2) and younger sister (L.N No 4) were also found to have abnormally low AHF activity/antigen ratios consistent with carrier status for the abnormal gene of classic hemophilia A.

Cytogenetic studies carried out in the patient revealed a single Barr body in 9*/ (normal 14–40*/) of cells obtained from buccal smears and in cultured lymphocytes consistently a karyotype of 46 chromosomes with 2 X-chromosomes. Thus, it seems reasonable to assume that the patient is homozygous for hemophilia A, i.e. that she inherited an abnormal X-chromosome from both her father who was a known bleeder and from her mother who was identified as a carrier This assumption is supported by the fact that the patient s grandmothers on the father's and mother s side had the same maiden name and lived in the same village in Northern Italy raising the strong possibility that they were related carriers. However the possibility cannot be excluded that our patient is a clinically affected carrier for classic hemophilia.

The rare occurrence of low levels of AHF in symptomatic heterozygotes for classic hemophilia has been explained on the basis of random inactivation of X-chromosomes, in excess of one, in all somatic cells at an early stage of embryogenesis [14]

The findings reported here illustrate the usefulness of quantitative estimation of AHF antigen in addition to AHF activity in the recognition of the carrier state for classic hemophilia A [2] Thus, whereas all females suspected to be carriers could be identified as carriers on the basis of a clearly abnormal AHF activity/AHF antigen ratio, two of these individuals (A. L., No. 2 An. L., No 10) had plasma AHF activity well within normal limits. However the finding on two occasions of a slightly abnormal AHF activity/AHF antigen ratio in our patient s sister-in-law (S. L., No. 6) who had a negative personal and family history for abnormal bleeding also indicates that as with any other laboratory test caution must

W thank Dr S. S. Sexnov, St. Louis Children' Hospital, for kindly performing these studies.

The template bleeding time was measured as previously described [18] Platelets in whole blood or PRP were counted by a semi-automated method (autocounter Technicon Corp Tarrytown, N Y) [6] The one-stage-prothrombin time [21] (using home-made human brain thromboplastin) activated partial thromboplastin time (aPTT) [20] (using DADE cephaloplastin), thrombin time [11] fibrinogen [7], clot urea solubility [9] and whole blood clot retraction [15] were performed according to standard methods. AHF activity was measured by a one-stage assay based on the aPTT [20] using diluted test plasma and plasma from a patient with severe classic hemophilia A Concentrations of AHF antigen were measured by an immunoelectric method recently described [1]. A first-stage *inhibitor assay* was performed by determining the aPTT on various mixtures of normal human pool plasma and patient plasma [10] *Platelet adhesiveness* was measured using a glass bead column retention method [5] Heparinized blood was left undisturbed for 30 min after venipuncture and mixed gently before passage through the glass bead column [8] *Platelet aggregation* was studied in citrated PRP stored at room temperature (platelet count adjusted to 250,000/mm³) by a turbidimetric technique [3] using a dual-channel aggregometer with recorder (Payton Associates, Inc., Buffalo, N Y.), as recently described [12], except that 0.45 ml of PRP and 0.05 ml of stimulus solution adenosine d phosphate (ADP Sigma Chemical Co St Louis, Mo.), epinephrine (EPI Sigma), acid-soluble collagen (COLL, Sigma) or Ristocetin® (RISTO Abbott Laboratories, North Chicago Ill.) were used. Final concentrations in PRP were ADP 3–5 μM EPI, 3–5 μM COLL, 8.5 μg/ml RISTO 0.9–1.2 mg/ml. Aggregation was judged to be normal with ADP and EPI if second-phase aggregation was clearly demonstrable and with COLL if the percentage change in optical density (PRP = 0, PFP = 100%) exceeded 70% at 5 min after the addition of the stimulus (based on observations on PRP from 25 normal individuals) Concentrations of vWF in plasma were determined by measuring Ristocetin induced aggregation of washed, formaldehyde-fixed human platelets in the presence of test plasma as compared to that observed with serial dilutions of normal human pool plasma [16]

Results and Discussion

The results of the hemostatic evaluation of the patient are shown in table I There was a mildly prolonged aPTT and moderate reduction in AHF activity (5%) The results of all other coagulation screening tests were normal and there was no evidence for a circulating anticoagulant The template bleeding time determined on three different occasions was within normal limits as were platelet adhesiveness and platelet aggregation in citrated PRP induced by ADP epinephrine, collagen and Ristocetin. These findings were not consistent with the previous diagnosis of vWD but they were consistent with that of moderately severe classic hemophilia A This diagnosis was further substantiated by the finding that the

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be exercised in the interpretation of borderline abnormal results, no matter how carefully the normal range has been determined.

The accurate classification of patients with mild to moderate deficiency of AHF activity is not simply of theoretical interest but has important practical implications in respect to the prevention and management of bleeding complications Thus, it is well known that patients with vWD may have an exaggerated or prolonged response to the infusion of plasma or plasma components as compared to patients with classic hemophilia A i.e., their requirements for plasma or plasma components may be less both in terms of total amount and frequency of administration Further although cryoprecipitate [22] and certain preparations of purified AHF [4 17 19 23] have been shown to correct the bleeding time and Ristocetin induced platelet aggregation in patients with vWD there is little or no published information available at present whether and to what extent this is true for the various commercially available AHF concentrates. In fact, we have observed minimal shortening of the bleeding time and rise in vWF activity in the presence of a marked rise in AHF activity in the plasma of four children with severe vWD after transfusion of high purity AHF concentrates obtained from different manufacturers. Thus, until reliable information in regard to the concentration of vWF activity in commercially available AHF concentrates and its effectiveness *in vivo* is available, fresh frozen plasma and cryoprecipitate appear to be the agents of choice in the management of patients with decreased vWF activity Finally because of the difference in the pattern of inheritance between the two disorders, differentiation between classic hemophilia and vWD is of crucial importance in respect to genetic counseling of patients and their relatives.

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Nonspecific Esterase Activity in 'Hairy Cells'[1]

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Key Words. Hairy cell leukemia · Nonspecific esterase activity · Monocytes

Abstract. Nonspecific esterase activity using α-naphthyl butyrate as substrate was found to be present in hairy cells from patients with hairy cell leukemia. Activity of this enzyme was markedly diminished and in some instances obliterated by sodium fluoride. Since α-naphthyl butyrate is believed to be a more specific substrate for monocytic-type nonspecific esterase than α-naphthyl acetate, its presence in hairy cells combined with fluoride inhibition which is also characteristic of monocytic nonspecific esterase provides additional evidence of monocytic properties of hairy cells.

This report describes the presence of fluoride-sensitive nonspecific esterase activity in 'hairy cells' using α-naphthyl butyrate as substrate. Since this substrate is believed to be more specific for cells of monocytic origin than α-naphthyl acetate [10], the presence of nonspecific esterase using α-naphthyl butyrate as substrate constitutes further evidence that the hairy cell possesses properties of monocytes.

Materials and Methods

Peripheral blood and marrow were obtained at the time of diagnosis from four patients with hairy cell leukemia. Marrow was also obtained at the time of diagnosis from a fifth patient with hairy cell leukemia. Imprints from cut sections of spleen were made from this patient at the time of necropsy. The diagnosis of hairy cell leukemia was made according to currently accepted clinical, morphological and cytochemical criteria [3, 9, 15, 16, 18]. Films of bone marrow flecks, peripheral blood, and spleen imprints were stained with Wright's stain for conventional light micros-

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Fig 2. Normal lymphocytes (arrows) and monocytes, showing intense nonspecific esterase activity in monocytes using α-naphthyl butyrate as substrates. Enzymatic activity could not be detected in virtually all lymphocytes. Sodium fluoride markedly diminished the enzymatic activity in monocytes. × 1,500.

be detected in these cells. Using α-naphthyl acetate as substrate, activity could be seen in hairy cells in all of the specimens. Enzymatic activity was markedly inhibited by addition of sodium fluoride to the incubation medium. Likewise, substantial nonspecific esterase activity was visualized when α-naphthyl butyrate was used as the substrate (fig. 1) and enzymatic activity was markedly inhibited, and in many instances obliterated, by addition of sodium fluoride.

In preparations of normal leukocytes, specific esterase activity was strong in granulocytes, and weak or absent in monocytes. Specific esterase activity could not be detected in lymphocytes. Nonspecific esterase activity using α-naphthyl acetate as substrate was weak in granulocytes and lymphocytes, and strong in monocytes. In all cells, activity of this enzyme was markedly reduced with the inclusion of fluoride in the incubation mixture. Using α-naphthyl butyrate as substrate for nonspecific esterase activity strong activity was present in monocytes, and weak in granulocytes. Fluoride markedly inhibited or obliterated this enzymatic activity. In lymphocytes, enzymatic activity could not be detected (fig. 2)



Fig 1 Group of hairy cells from splenic imprint, showing punctate intense non specific esterase activity using α-naphthyl butyrate as substrate. × 1,500. Insets: Hairy cells, peripheral blood, showing punctate nonspecific esterase activity using α-naphthyl butyrate as substrate. In all instances, enzymatic activity was markedly diminished or obliterated by sodium fluoride. × 1,500

copy Films were also stained for tartrate-resistant acid phosphatase [18], and for specific esterase using naphthol ASD-chloroacetate as substrate [16]

Separate specimens were stained for nonspecific esterase activity using α naph thyl acetate in one instance, and α-naphthyl butyrate in another [10, 17] Simultaneously duplicate coverslips were stained for nonspecific esterases using these substrates, but in the presence of sodium fluoride

α-Naphthyl butyrate is thought to be a more specific substrate for monocyt c type nonspecific esterase than α-naphthyl acetate [10] Inclusion of sodium fluoride in the incubation medium is believed to further enhance the specificity of the reac tion for monocytic type nonspecific esterase, which is markedly inhibited by fluoride [4] Consequently both α-naphthyl butyrate and fluoride were utilized to ascertain whether the hairy cell possessed properties similar to those found in monocytes [10, 17]

Results

Acid phosphatase activity was resistant to tartrate, a property believed to be characteristic of hairy cells [18] Specific esterase activity could not

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Discussion

Controversy surrounds the origin of the hairy cell. Some investigators believe that it is of lymphocytic derivation [1–3, 5, 7, 11, 13, 14], whereas others hold the opinion that is closely related to monocytes and reticulum cells [6, 8, 9, 12, 15, 16]. Classification of leukemias has been improved with the use of esterase reactions [10, 17]. Specific esterase activity using naphthol ASD-chloroacetate as substrate is typical of cells of granulocytic origin, whereas nonspecific esterase activity inhibited by fluoride is characteristic of cells of monocytic or reticulum cell origin [10, 17].

In the present study, hairy cells possessed nonspecific esterase activity when α-naphthyl acetate was used as substrate, as shown in earlier studies [9, 15] and confirmed recently [12]. This report extends these observations by demonstrating that the nonspecific esterase activity using α-naphthyl acetate is fluoride-sensitive, as is monocytic nonspecific esterase using this substrate.

The results demonstrate further that in hairy cells, nonspecific esterase activity using α-naphthyl butyrate as substrate is also markedly inhibited by fluoride. Recent cytochemical and electrophoretic studies have shown that α-naphthyl butyrate may be a more specific substrate for monocytic-type nonspecific esterase than α-naphthyl acetate [10]. The presence of strong fluoride-sensitive nonspecific esterase activity in normal monocytes and virtually absent activity in normal lymphocytes using α-naphthyl butyrate as shown in the present study, reinforces this concept. Accordingly, the findings in this report are consistent with evidence obtained by others that hairy cells possess properties of monocytes and reticulum cells.

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until the possibility of rapidly killing the leukemic blast cells became available [1] thus, the complete remission (CR) rate at St. Louis Hospital rose from 11% before 1967 to 43% after 1967 when daunomycin was first used. However subsequent reports [3–5 7 9 13] have not confirmed these results, providing a lower CR rate ranging from 0 to 33 %.

During the last 5 years, 13 adult patients with APL have been admitted to our institution, of whom 7 have attained CR, a proportion similar to that achieved by BERNARD et al. [1] We report here on the clinical and laboratory data pertaining to these patients, on the therapy and on the course of the disease after remission induction.

Patients and Methods

From January 1972, to September 1976, 84 adult patients with AML were first admitted and diagnosed at our institution. 13 of them, or 15.4%, had diagnosis of APL, based on the characteristic morphology of leukemic blast cells at bone marrow examination [1, 8]. Details of these patients are reported in table I. There were 7 males, and 6 females. Median age was 35 years (16–69 years). The median time lapse from the first symptoms to diagnosis was 20 days (7–60 days) All the patients but one (case 11) presented with hemorrhages. All the patients but two (cases 2 and 11) were febrile at diagnosis. No patient had spleen, liver or lymph node enlargement. Results of hematological examination and appropriate blood clotting tests, are also listed in table I.

The fibrinogen level was determined by the heat precipitation technique or by radial immunodiffusion (normal values 150–400 mg/100 ml) The fibrin-fibrinogen degradation products (FDP) level was determinated by Thrombo-Wellcotest (normal values. <10 µg/ml).

The protocol of therapy used for remission induction is shown in table II. Chemotherapy was always started on the first day of admission, provided that the patient had not yet developed fatal (brain) hemorrhage. Simultaneously all patients were given platelet transfusions (3–6 U daily or every other day) for as long as was needed. The bacterial flora of the mouth, throat, sputum, and urine was monitored with appropriate cultures, weekly or more frequently if clinically indicated. Repeated blood cultures were taken at the beginning of each febrile period. Antibiotics were given only if the patient was febrile standard regimen included carbenicillin (0.45 g/kg/day), plus gentamycin (3 mg/kg day) and cephalotin (90 mg/kg/day) given intravenously as boost every 8 h. This standard regimen was modified thereafter according to the results of appropriate cultures. The patients were kept in normal open wards, received the normal hospital diet, and were not given nonadsorbable antibiotics orally. The mouth was washed very frequently with chlorohexidine and nystatin. No patient received granulocyte transfusions.

CR was defined as complete normalization of marrow blood, and blood clotting tests, and was checked every month after remission induction. Remission main-

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Acute Promyelocytic Leukemia Results of Therapy and Analysis of 13 Cases[1]

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Key Words. Acute promyelocytic leukemia Acute myeloid leukemia Daunomycin Platelet transfusion

Abstract Acute promyelocytic leukemia (APL) was diagnosed in 13 of 84 adult patients (15.4%) with acute myeloid leukemia (AML) first admitted between 1972 and 1976. All patients had clinical and/or laboratory evidence of defibrination syndrome. Four patients died of cerebral hemorrhage within 2 days of admission. Two patients died of generalized infection on days 7 and 16, respectively after admission. The remaining 7 patients (54%) underwent complete remission (CR) with daunomycin, arabinosyl cytosine, and adriamycin. All patients received massive platelet transfusion, no heparin, and no granulocyte transfusion. CR was more frequent in patients with a very low blast cell count and a fibrinogen level higher than 100 mg/100 ml. Median survival of these seven CR patients with APL is similar (15 months) to that of CR patients with other types of AML treated at the same institution during the same period.

Amongst acute myeloid leukemias (AML) acute promyelocytic leukemia (APL) is a distinct entity due to the morphology of the leukemic blast cells, and their peculiar property of triggering an intravascular coagulation or defibrination syndrome leading to early and very severe hemorrhages [1 6 8 12] The course of the disease was very rapid, and terminated in a few days with a fatal hemorrhage more often in the brain

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until the possibility of rapidly killing the leukemic blast cells became available [1] thus, the complete remission (CR) rate at St. Louis Hospital rose from 11% before 1967 to 43% after 1967 when daunomycin was first used. However subsequent reports [3–5 7 9 13] have not confirmed these results, providing a lower CR rate ranging from 0 to 33%.

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Table I Clinical and laboratory details, outcome, and course of the 13 patients with APL

Patient		Sex	Age years	Time lapse from first symptoms to diagnosis, days	Presenting symptoms	Fever °C	Hb g/100 ml	PMN $\times 10^3/\mu l$
1	R.P	M	32	16	hematuria, epistaxis, petechiae, gingival bleeding	<38	12.1	0.7
2	C.M L.	F	16	30	hematomas, petechiae, menorrhagia	no	5 0	0.5
3	N A.	M	56	10	hematomas, petechiae, epistaxis, gingival bleeding	>38	9 6	0.5
4	A.F	F	69	30	hematomas, petechiae, gingival bleeding	>38	8.1	0.7
5	V S.	M	55	8	melena, epistaxis, hematuria, hematomas	>38	10.3	0.5
6	Z.L.	M	64	30	hematuria hematomas, epistaxis, gingival bleeding	<38	8 9	0.9
7	G P	M	27	15	hematuria, hematomas, petechiae, gingival bleeding	>38	11.2	0.3
8	B.N	F	45	60	menorrhagia petechiae	<38	3 4	0.3
9	G L.	F	20	20	menorrhagia hematuria, hematomas, gingival bleeding	<38	11 8	0.7
10	M F	M	35	[illegible]0	hematuria hepistaxis, hematomas, gingival bleeding	>38	8 7	0.7
11	P P	F	32	38	mild asthenia	no	11 4	0 6
1	F G	M	28	7	petechiae, gingival bleeding	<38	14 0	1 5
13	M D	F	55	10	hematuria hematomas, petechiae, gingival bleeding	>38	10 0	0.5

+ = The patient is in continuous CR ND = not done.

Table I (continued)

Blast cells $10^3/\mu l$	Platelets $10^3/\mu l$	Fibrinogen mg/100 ml	FDP $\mu g/ml$	PTT sec	Pro-thrombin %	Complete remission duration, months	Course
5.2	41	160	ND	45	59		death on day 2, brain hemorrhage
3.5	12	90	60	29	57		death on day 1 brain hemorrhage
1.3	15	90	100	33	60		death on day 2, brain hemorrhage
20.0	20	40	120	53	33		death on day 2, brain hemorrhage
6.2	50	45	200	39	43		death on day 7 infection
4.0	54	40	80	38	40		death on day 16, infection
10.3	25	150	400	37	31	1.6	survival 7.2 months, death during second relapse, brain hemorrhage
0.1	73	135	ND	27	100	7.6	survival 15.8 months, death during second relapse, infection
0.9	48	115	2	32	100	4.9	survival 9.8 months, death during first relapse, infection
0.4	33	205	32	36	59	16.0+	still in first CR
0.0	132	40	25	35	70	9.0+	still in first CR
0.7	14	130	40	30	100	[illegible]4	still in first CR
14.8	21	160	60	34	60	1.0+	still in first CR

Table II Protocol of therapy used for remission induction in APL

(1) Daunomycin, 1 5 mg/kg i v × 3–4 days

↓

5 to 10-day interval

↓

(2) ARA-C 10 mg/kg i.v in continuous perfusion over 24 h, followed by daunomycin 1 mg/kg i.v and adriamycin 1 mg/kg i v

↓

10 to 15-day interval

↓

(3) ARA-C 1 mg/kg/8 h i.v as a booster × 3 days, followed by daunomycin 1 mg/kg i.v

↓

10-day interval

↓

(4) ARA-C 1 mg/kg/8 h i v as a booster × 3 days, followed by daunomycin 1 mg/kg i v

tenan e included a 5-day course of arabinosylcytosine (ARA-C) + thioguanine (3 mg/kg/12 h each) given every 40 days, and cyclic injections of BCG (Glaxo) and irradiated allogenic leukemic blast cells, according to the technique described by POWLES *et al* [10]

Results

The outcome of therapy is as follows. Four patients (30 8%) devel oped cerebral hemorrhage a few hours after admission and died within 2 days from diagnosis, without receiving chemotherapy Two patients (15 4%) died 7 and 16 days repectively after admission with severe generalized infection they had received one and two cycles of chemo therapy and 21 and 53 platelet units, respectively At the time of dying, their hemorrhagic syndrome was well controlled The remaining 7 patients (53 8%) underwent CR. Table III lists the details of the therapy and of the clinical course of these 7 patients All of them but one received the four courses of chemotherapy scheduled Most of the platelet transfu sions were given during the first 2 weeks from admission, but many pa tients requested platelet support also later The median number of days spent with hemorrhagic manifestations was 8 with a wide range from 0

Table III. Details on the therapy and the course of the 7 patients who underwent CR

Cycles of chemotherapy	extreme	3–4
	median	4
Platelet units given during the first 2 weeks	extreme	13–55
	median	33
	total	209
Platelet units given thereafter	extreme	0–24
	median	12
	total	81
Days with hemorrhages	extreme	0–39
	median	8
Febrile episodes	extreme	1–4
	median	2
	total	13
Days with fever	extreme	13–28
	median	16
	total	128
Days to remission	extreme	25–60
	median	38
Days in hospital	extreme	30–73
	median	52
	total	374

(patient 11) to 35 days in patient 9 (who had prolonged thrombocytopenia), and 39 days, respectively in patient 13 (who continued for many days to have gross hematuria, despite of normal platelet count, blood clotting tests, and FDP level). All the patients experienced at least one severe febrile episode, and the median number of days spent with fever per patient, was 16 days. Median interval from admission to CR was 38 days, and the median number of days in the hospital was 52 days.

Figures 1 and 2 show the distribution of the 6 patients who died early and of the 7 patients who underwent CR, according to several clinical and laboratory parameters measurable at diagnosis. The number of patients in each group is too small to find any correlation. Nevertheless, it appears that the distribution according to the duration of the prediagnostic phase, the Hb level, and the number of polymorphonuclear neutrophilics (PMN) in peripheral blood (fig. 1) is identical in the two groups. Also age distribution (fig. 1) is not different, but it appears that only 1 of 5 patients

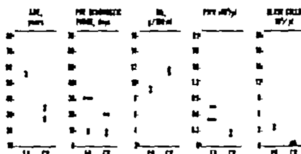


Fig 1 Distribution of the patients, who had an early death (ED), or underwent CR, according to the age, the length of the prediagnostic phase, the Hb level, and the number of PMN and blast cells in the peripheral blood, at diagnosis.

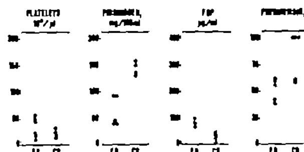


Fig 2. Distribution of the patients who had an early death (ED) or underwent CR, according to the platelet count, the fibrinogen and FDP level and the prothrombin activity at diagnosis.

above 50 underwent CR, versus 6 of 8 patients under 50 The distribution according to blast cell count (fig. 1) is interesting showing that 5 of 7 CR were obtained in patients with a blast cell count lower than 1 0 × $10^3/\mu l$ Figure 2 shows no correlation with the platelet number while the patient distribution according to the results of blood clotting tests is apparently different in the two groups. The fibrinogen level was above 100 mg/100 ml in 6 of the 7 patients who underwent CR, versus 1 of the 6 patients who died early The FDP level was higher than 60 μg/ml in 1 of the 6 patients who achieved CR, versus 5 of the 5 patients who died early Prothrombin activity was lower than 60% in all the patients who died early while in 3 of the 7 CR patients it was normal.

Figure 3 shows platelet and PMN counts, in the interval between diagnosis and marrow and blood CR. The platelet number was nearly always

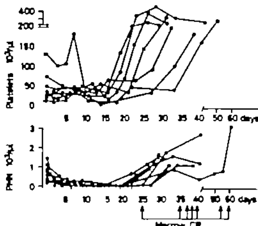


Fig 3 Behavior of platelet and PMN count during remission induction, in the 7 patients who underwent CR. All patients received platelet transfusions, mostly between day 0 and day 15.

kept between 20 and 40 × $10^3/\mu l$ by means of platelet transfusion from diagnosis to day 15 Spontaneous platelet recovery appeared betwen day 15 and day 35 The PMN count was practically zero for a long period after diagnosis. 20–27 days. As previously shown in table III this long period of agranulocytosis was complicated by a number of severe, febrile, infectious episodes.

Discussion

The frequency of APL, amongst all AML, calculated on the basis of 820 cases of AML, is 6.8% [2, 4 5 9 13] ranging from the 6.2% reported by WALLACE *et al.* [13] to the 15 0% found by BRUN *et al* [2] At our institution, the frequency of APL was of 15 4 %, approaching the latter value. Therefore, APL is a rare type of leukemia, but deserves separate consideration for its fulminant course, its constant defibrination syndrome, and its peculiar cellular morphology APL is then different from other AML, and requests a somewhat different therapeutic approach, in order that the patients may survive the hemorrhagic complications of the first few days [8] The importance of the therapy for the fate of APL patients was emphasized by BERNARD *et al* [1] who reported 4/36 CR

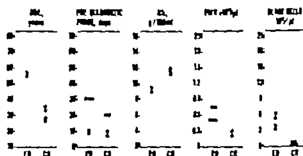


Fig 1 Distribution of the patients, who had an early death (ED), or underwent CR, according to the age, the length of the prediagnostic phase, the Hb level, and the number of PMN and blast cells in the peripheral blood, at diagnosis.

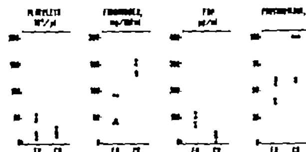


Fig 2 Distribution of the patients who had an early death (ED), or underwent CR, according to the platelet count, the fibrinogen and FDP level, and the prothrombin activity at diagnosis.

above 50 underwent CR, versus 6 of 8 patients under 50 The distribution according to blast cell count (fig 1) is interesting, showing that 5 of 7 CR were obtained in patients with a blast cell count lower than $1.0 \times 10^9/\mu l$. Figure 2 shows no correlation with the platelet number while the patient distribution according to the results of blood clotting tests is apparently different in the two groups. The fibrinogen level was above 100 mg/100 ml in 6 of the 7 patients who underwent CR, versus 1 of the 6 patients who died early The FDP level was higher than 60 μg/ml in 1 of the 6 patients who achieved CR, versus 5 of the 5 patients who died early Prothrombin activity was lower than 60% in all the patients who died early while in 3 of the 7 CR patients it was normal

Figure 3 shows platelet and PMN counts, in the interval between diag nosis and marrow and blood CR. The platelet number was nearly always

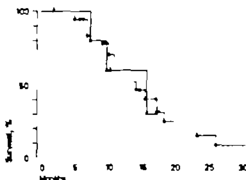


Fig 4 Actuarial survival of CR patients with APL (▲ n = 7), and of CR patients with other types of AML (O = 22).

APL may be drastically modified by using prompt and generous platelet support and by starting chemotherapy immediately with an adequate amount of daunomycin. We cannot conclude whether adding heparin to the above therapy would have provided better results, though the brain hemorrhages, which caused the death of 4 patients, developed in so few hours so as to make it doubtful. We also doubt whether heparin would have been useful in shortening the duration of the hemorrhagic syndrome in the patients where it lasted for many days. Actually in these cases the hemorrhages were mild, and correlated better with the platelet level (low), than with fibrinogenemia (normal) or the FDP level (low).

All our patients developed at least one, and more frequently two or three severe, febrile, infectious episodes and the total number of days spent with fever was 128 or 34 / of all the days spent in the hospital by the 7 CR patients. Thus, the frequency of infectious complications was higher than in patients with AML treated at our institution, confirming that prolonged agranulocytosis (fig. 1), possibly related to daunomycin administration, is an important factor that may limit the CR rate. In effect, 2 patients who survived the first days, and whose hemorrhagic syndrome was well controlled, died because of infection. Both patients were more than 50 years old.

Analysis of this group of patients for a search of prognostic parameters has pointed to a negative correlation with the blast cell count, and to a positive correlation with the fibrinogen level, as previously observed by

Table IV Literature data on CR rate in APL

Reference	Period	Number of patients	CR
BERNARD *et al* [1]	1967–1973	44	19 (43%)
BRUN *et al* [2]	?–1973	12	6 (50%)
CACCIOLA *et al* [3]	?–1975	7	0
CLARKSON *et al* [4]	1970–1973	6	0
CROWTHER *et al* [5]	1969–1973	6	2 (33%)
FUSCO *et al* [7]	?–1975	6	0
EORTC [9]	1970–1971	11	1 (10%)
WALLACE *et al* [13]	1963–1973	31	5 (16%)
Total		123	33 (27)

(11%) before 1967 and 19/44 CR (43%) after 1967 These authors at tributed such a marked increase of the CR rate to the immediate adminis tration of large doses of daunomycin (2 mg/kg/day in 4-day courses repeated at 3-day intervals) leading to rapid leukemic cell death. To our knowledge there are no published reports on APL, based on a number of observations comparable to that of BERNARD *et al* [1] Moreover the CR rate observed by others was consistently lower as compared to BERNARD *et al* [1] (table IV) with the exception of the patients treated at Creteil and reported in late 1973 by BRUN *et al* [2] who received daunomycin during the first 3 days, at a dose of 100 mg/m²/day

The main cause of death in all reported series is hemorrhage. Massive platelet transfusion and prompt administration of adequate doses of dau nomycin may effectively control and eliminate the defibrination syndrome [1 2, 6 8 11] allowing the patients to receive further chemotherapy and to undergo CR The use of heparin though apparently logical [8] does not seem to add significantly to platelet transfusion and daunomycin [1 3 6 7 11] The usefulness of replacement therapy with fibrinogen and other blood clotting factors cannot be assessed on the basis of published data. The administration of large doses of daunomycin is obviously fol lowed by a prolonged and severe marrow aplasia that may be responsible for death later during remission induction 9 of 33 (27%) patients treated by BERNARD *et al* [1] died in that period, because of septicemia

Our results are similar to those of BERNARD *et al* [1] and BRUN *et al* [2] supporting the conclusion that the spontaneous fulminant course of

Thioguanine, or CAR and daunorubicin) and maintenance therapy (by CAR or methylgaz) in acute myeloid leukemia. Biomedicine *18.* 192 198 (1973).

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BERNARD *et al* [1] Our data suggest that also the FDP level and prothrombin activity may be of some importance.

The data published by BERNARD *et al* [1] suggested that CR duration and survival of APL patients are probably longer than those of other AML patients median CR duration of the patients of BERNARD *et al* [1] was 26 months, and 4 patients were in their first CR 4 years after diagnosis. The same was not observed by BRUN *et al* [2] as 4 of their 6 CR patients relapsed within 16 months. Also in our institution survival of CR patients with APL is identical to that of CR patients with other types of AML treated during the same period (fig. 4)

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Multiple Myeloma Associated with Kaposi Sarcoma

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Key Words. Multiple myeloma Kaposi sarcoma Melphalan

Abstract A patient with multiple myeloma (MM) who developed Kaposi sar coma (KS) is described. The KS appeared 18 months after the diagnosis of MM and 1 month after the treatment was changed from cyclophosphamide to melphalan. The treatment with melphalan was discontinued and the spread of the KS was ar rested by irradiation and bleomycin. One month after the melphalan was restarted, the KS advanced The patient died 28 months after the diagnosis of MM and 10 months after KS had developed.

The association of KS and MM is discussed and the previously reported cases are reviewed A possible connection between the treatment with melphalan and the development of KS is proposed.

Over 100 years have passed since the original description of Kaposi s sarcoma (KS) but the origin of KS cells is still uncertain [1] Reviews of large series of patients with KS [10 13] have established the association of KS with a second neoplasm particularly lymphoreticular such as Hodgkin s disease, lymphosarcoma and lymphatic leukaemia. The reports on KS with multiple myeloma (MM) and other plasma cell dyscrasias are rare and we would like to present an additional case with this association.

Case Report

A 59-year-old male, born in Poland, was referred to the Hospital in October 1973, because of weakness and bone pain of 6 months duration. Physical examina tion was without any pathological findings.

[1] Established investigator of the Chief Scientist s Bureau, Ministry of Health, Israel.

The laboratory examinations disclosed the following findings. ESR 117/120, haemoglobin 11.8 g/100 ml, haematocrit 36% white blood cells 8,200/μl, with 46% neutrophils, 1% band forms, 2% eosinophils, 1% basophils, 7% monocytes, and 43% lymphocytes. Platelet count was 269,000/μl. Total serum proteins were 11 g/100 ml, albumin 4.4 g/100 ml and globulin 6.6 g/100 ml. Paper electrophoresis of the serum protein showed albumin 31%, alpha₁ 5%, alpha₂ 7%, gamma 46% with a monoclonal peak. Immunoelectrophoresis of the serum proteins revealed IgG 9,200 mg/100 ml, IgA 20 mg/100 ml, IgM 25 mg/100 ml. The light chain was lambda. 24-hour excretion of protein in the urine was 1.6 g, Bence Jones positive. Its immunoelectrophoresis showed IgG 770 mg/100 ml lambda. Blood glucose, urea, creatinine, uric acid, sodium, potassium, chloride, calcium, phosphate, alkaline phosphatase, SGOT SGPT prothrombin time, fibrinogen and bilirubin were normal. Bone marrow aspiration biopsy revealed clusters of immature plasma cells (fig. 1). X-ray examination of the bones disclosed lytic lesions in the dorsal vertebra, ribs and the occipital region of the skull.

The patient was diagnosed to suffer from multiple myeloma and treatment with 50 mg/day cyclophosphamide and 20 mg/day prednisone was started. One month later he complained of watery and occasionally bloody diarrhoea which partially responded to symptomatic treatment. The therapeutic schedule was continued for 18 months without significant clinical improvement. The blood protein level did not decrease.

In March 1975 the treatment was replaced by melphalan 0.15 mg/kg and prednisone 40 mg/day 4 days every 6 weeks, and allopurinol 300 mg/day because of hyperuricaemia. One month later the patient noticed blue patchy infiltrations on his feet. Over the next 5 months these infiltrates and purple nodules continued to spread proximally over the legs up to the groin, and were associated with pain and oedema (fig. 2).

The patient was hospitalized in August 1975. On physical examination, in addition to the skin nodules, [illegible] heares were heard in both lungs and the liver edge was palpable. Except for 9.5 g/100 ml haemoglobin, the rest of the laboratory tests showed no change. A biopsy of one of the nodules was performed. On histological examination the subendoderm was found infiltrated with fibroblasts arranged in strands. Among [illegible]hem were seen atypical cells and few mitotic figures. Deposits of haemosiderin and [illegible]ncreased vascular foci were found. These findings were compatible with Kaposi [illegible]rcoma with fibroblastic changes (fig. 3, 4).

Electron Microscopy

[illegible] mall piece of the skin tumour was immediately fixed in cold 1 [illegible] gl[illegible] [illegible]ldehyde in phosphate buffer pH 7.4 post-fixed in osmium tetrox[illegible] [illegible]d[illegible] [illegible]d[illegible] hydrated in graded alcohols and embedded in Epon 812. Thin sec[illegible] [illegible] [illegible]w[illegible] cut with an LKB Ultratome III and examined with a Philips 300 electron microscope.

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Fig 3 Fibroblasts arranged in strands, among them atypical and mitotic figures. HE. × 100.

Fig 4 Increased vascular formation in the tumour tissue. HE. × 360.

Most of the cells possessed an oval nucleus with an irregularly shaped nuclear membrane. The heterochromatin was scanty (fig. 5), or represent ed in small amounts in the vicinity of the nuclear envelope (fig. 6). The amount of cytoplasm was usually small, with poorly presented organelles, although in a few instances cells with lower nucleo-cytoplasmic ratio were found In these cases, abundant rough endoplasmic reticulum was seen (fig. 6) The cells were surrounded by huge amounts of collagen, easily identified by its typical structure.

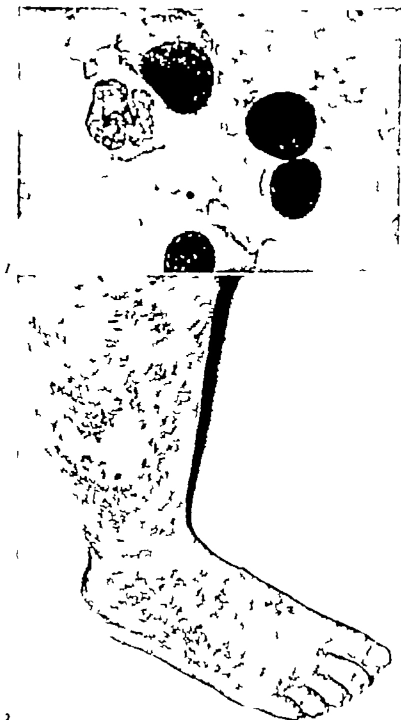


Fig 1 Bone marrow smear showing myeloma cells. May-Grünwald-Giemsa. × 1,000.

Fig 2 Nodular skin lesions of Kaposi's sarcoma on the legs.

The patient was irradiated with 1,000 R to the soles and later was given bleomycin 30 mg i.v. once a week for 10 weeks. Consequently the pain and the progression of the sarcoma subsided. The treatment with bleomycin was complicated by fever up to 40 °C, eosinophilia up to 20% and pulmonary infiltrates which disappeared after cessation of the therapy. At that time plasma cells and eosinophils were found in the urine sediment.

One month after the last bleomycin injection and the readministration of melphalan 2 mg/day, the KS nodules spread over the patient's abdomen, hands and soft palate. He was treated by irradiation of the skin nodules.

The patient was hospitalized again in January 1976 because of collapse of L_1 vertebra and pneumonia. He developed acute renal failure and died. Permission for a post-mortem examination was not granted.

Discussion

The clinical and laboratory findings in our patient including the monoclonal immunoglobulin IgG with light lambda chain, the immature plasma cell infiltration of the bone marrow and the lytic bone lesions were consistent with the diagnosis of multiple myeloma. The appearance of the skin lesions and their histological features were typical for KS.

The origin of the tumor cells in cases of KS is not clear. PEPLER and THERON [12] on the basis of the electron microscope observations on four patients with KS, concluded that the tumour cells originate from the Schwann cells. On the other hand, HASHIMOTO and LEVER [4] defined two types of tumour cells – endothelial cells and phagocytic fibroblasts. The latter type appeared to originate from the perithelial cells of proliferating vessels. ORBANEJA *et al.* [11] distinguished three forms of KS – compact cellular, cavernous and mixed types. The first type was composed of endothelial cells and pericytes, fusiform cells, most probably fibroblasts, and histiocytic cells containing phagocytized red blood cells.

Fig. 5. A tumour cell showing irregular nucleus, scanty heterochromatin, abundant cytoplasm with lamellae of endoplasmic reticulum, some of them dilated. ×9,850

Fig. 6. A fusiform tumour cell with high nucleo-cytoplasmic ratio, surrounded by numerous collagen fibres. ×10,000.

5

6

telnaemia [2] polyclonal hypergammaglobulinaemia [15] and macroglobulinaemia [6]

The cause for the association of KS with multiple myeloma and other lymphoreticular neoplasms is not clear It is possible, as suggested by WARNER and O'LOUGHLIN [16] that KS is the result of chronic interaction of altered lymphocytes of the lymphoreticular process with normal lymphocytes. This interaction causes the liberation of an angiogenesis factor and an induction of an oncogenic virus, and finally the development of KS.

In our patient the first signs of KS appeared 1 month after treatment with melphalan was started. The drug was discontinued while the patient was treated for the KS. One month following the readministration of melphalan, the KS started to spread rapidly Similarly Mazzaferri's patient was treated with melphalan and prednisone and developed KS 3.5 months later

MYERS *et al* [9] reported two kidney-transplanted patients who were treated continuously with prednisone and azathioprine and who developed KS. In one of the patients the KS disappeared within 2 months after cessation of the azathioprine and in the second one, 2 months after the removal of the transplant and discontinuation of all immunosuppressive drugs.

These observations raise the possibility that the immunosuppressive therapy depressed the immune surveillance and allowed the development of the KS.

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Table I Cases of Kaposi's sarcoma associated with multiple myeloma

Author	Age	Sex	Hb g/100 ml	Hepato-megaly	Ig type	Bone le-sions	Order of diagnosis	Survival after the diagnosis of the second malignancy
RAYNOLDS *et al.* [13]	57	M	10.	–	ND	+	MM diagnosed 8 years after KS	4 months
GELLIN [3]	77	M	6.0	+	ND	ND	MM and KS diagnosed at the same time	ND
MAZZAFERRI and PENN [7]	63	M	4 8	+	IgA K	–	KS diagnosed 3.5 m after MM	10 months
LAW [5]	74	M	9.5	–	IgG K	–	MM and KS diagnosed at the same time	Alive when reported
MANDEL *et al* (present study)	59	M	9.5	+	IgG	+	KS diagnosed 18 m after MM	10 months

ND = No data available.

The ultrastructural appearance of the tumour cells in our case was suggestive of fibroblasts The large amount of collagen supports this view The finding of different types of cells in previously reported cases suggests that there are several stages in the development of KS the latest being the stage of fibroblastic cell type

The association of KS with other neoplasms such as Hodgkin s disease, lymphoma, lymphatic leukaemia, thymoma and carcinoma is well established [8 10 13]

In most of the cases, KS is associated with diseases of the lymphoreticular system. Since the plasma cells are supposed to be derived from the lymphocytes, it is conceivable that KS also would be associated with plasma cell dyscrasia A review of the literature revealed five additional cases of KS associated with plasma cell dyscrasia [3 5 7 10 13] Some of the clinical features of four patients and our own case are given in table I

In addition to MM KS was reported to be associated with other paraproteinaemias such as monoclonal M component [14] Bence Jones pro-

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Thrombocytopenic Purpura as the Sole Manifestation of Recurrence in Hodgkin s Disease

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Key Words. Thrombocytic purpura Hodgkin disease ITP

Abstract A patient with Hodgkin disease is described, in whom thrombocytopenic purpura was the presenting manifestation of recurrence 15 years after the initial manifestation of the disease. Diverse factors involved in the mechanism of thrombocytopenia in Hodgkin disease are discussed.

Only a few well-documented cases of Hodgkin s disease with thrombocytopenia have been described in the literature [1 3–5 8 9] In this report we describe a patient in whom Hodgkin s disease was diagnosed 15 years previously and in whom thrombocytopenia was the sole manifestation of recurrence of the disease.

Case Report

A 23-year-old bedouin male was admitted to the hospital in August, 1972 because of epistaxis and weakness during the past three weeks. I October 1957 a diagnosis of Hodgkin disease was made on lymph node biopsy from the neck The patient was treated with nitrogen mustard I September 1958 and February 1960 he was treated by radiotherapy (total 3,100 rad) to the neck for recurrent enlargement of lymph nodes. Since 1960 until the present admission no follow-up was recorded and the patient lived together ith his tribe in the Negev area. On admission he was pale and physically underdeveloped The pulse rate was 140/min, temperature 36.8 °C and blood pressure 130/80. A large postirradiation scar was evident in the neck. Shotty lymph nodes were palpable in the groin. The spleen was enlarged and palpable 4 cm below the left costal margin.

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Only a few well-documented cases of Hodgkin's disease with thrombocytopenia have been described in the literature [1, 3–5, 8, 9]. In this report we describe a patient in whom Hodgkin's disease was diagnosed 15 years previously and in whom thrombocytopenia was the sole manifestation of recurrence of the disease.

Case Report

A 23-year-old bedouin male was admitted to the hospital in August, 1972 because of epistaxis and weakness during the past three weeks. In October 1957, diagnosis of Hodgkin's disease was made on a lymph node biopsy from the neck. The patient was treated with nitrogen mustard. In September 1958 and February 1960 he was treated by radiotherapy (total 3,100 rad) to the neck for recurrent enlargement of lymph nodes. Since 1960 until the present admission no follow-up was recorded and the patient lived together with his tribe in the Negev area. On admission he was pale and physically underdeveloped. The pulse rate was 140/min, temperature 36.8 °C and blood pressure 130/70. A large postirradiation scar was evident in the neck. Shotty lymph nodes were palpable in the groin. The spleen was enlarged and palpable 4 cm below the left costal margin.



Fig 1 Lymph node biopsy showing Hodgkin's disease nodular sclerosing type. HE. × 125

Laboratory Findings

ESR 164/171 mm (Westergren), hemoglobin 4,2 g%, reticulocytes 5%, leukocytes 3,800/mm³ differential count 71% polymorphs, 1 basophil, 20% lymphocytes and 8% monocytes. The peripheral blood smear showed a picture of dimorphic anemia and thrombocytopenia. Platelet count 6,000/mm³ serum, urea, creatinine, sugar bilirubin, alkaline phosphatase, SGOT SGPT and prothrombin time were normal. Protein electrophoresis showed an elevation of the α_2-globulin, but immunoelectrophoresis was normal. LE cells were not found and tests for ANA (antinuclear antibody) were negative. Coombs test was negative. Serum iron was 18 µg/100 ml (normal 65–120 µg/100 ml) and whole blood folic acid 75 ng/ml (normal 100 ng/ml). Bone marrow aspiration revealed a hypercellular marrow. There were few giant stabs, macroblasts and pronormoblasts, a small number of megaloblasts and a normal number of megakaryocytes, but no budding of platelets was present. Prussian blue staining did not reveal any iron deposits. Chest X-ray was normal. Because of the serious condition of the patient therapy with blood and platelet transfusions and prednisone 60 mg/day was started. The body temperature remained between 37.5 and 38 °C in spite of the therapy with steroids. Repeated blood cultures, chest roentgenograms, intravenous pyelography and X-ray of the gastrointestinal tract were negative. The number of platelets increased gradually. In October 1972, the platelet count was within normal values, and thereafter it persisted in the normal range. A liver biopsy was normal and one of the groin lymph nodes revealed a nonspecific histological picture. Lymphography performed on in January 1973

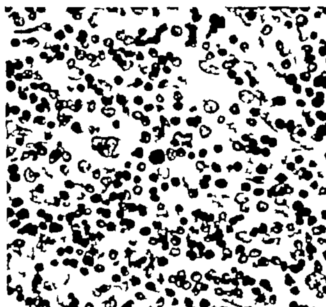


Fig 2 High power view of one of the nodules, showing Reed-Sternberg cells. HE. × 500.

showed involvement of the para-aortic lymph nodes. Platelet count was 262,000/mm   hen laparotomy and splenectomy were performed. Gastrohepatic, pericoeliac mesenteric and para-aortic lymph nodes were taken for examination and an open liver biopsy was performed. The para-aortic and pericoeliac lymph nodes showed the picture of Hodgkin disease: nodular sclerosing type with dense intersecting collagen bands and lacunar cells (fig. 1, 2). I   node from the splenic hilum the picture corresponded to the mixed type of Hodgkin disease. Mesenteric lymph nodes showed sinus hyperplasia and no evidence of Hodgkin disease. The spleen weighed 900 g, had   congested red-brow cut surface, and showed many scattered whitish foci which showed the histological picture of Hodgki   disease. The red pulp was congested, the sinusoidal lining cells were swollen and there was moderate hemosiderosis. There was no evidence of extramedullary hematopoiesis. One section of spleen showed   fairly large recent infarct. A liver biopsy showed no evidence of Hodgkin disease. Blood counts 2 days fter splenectomy ere Hb 10.8 g%, Ht 37% RBC 3.8 10⁶ mm³ reticulocytes 6%, WBC 22,000/mm   polymorphs 93%, bandforms 5% lymphocytes 1   and monocytes 1 %, platelets 380,000 mm³. After laparotomy   regimen of chemotherapy (MOPP) wa started. The patient developed   sever respiratory infection and was treated with antibiotics (first erythromycin, followed later by lincocin, septrin and orbenil).



Fig 1 Lymph node biopsy showing Hodgkin s disease nodular sclerosing type HE. × 1.5

Laboratory Findings

ESR 164/171 mm (Westergren) hemoglobin 4,2 g%, reticulocytes 5% , leukocytes 3,800/mm differential count 71%/ polymorphs, 1 / basophil, 20%/ lymphocytes and 8%/ monocytes. The peripheral blood smear showed a picture of dimorphic anemia and thrombocytopenia. Platelet count 6,000/mm³ serum, urea, creatinine sugar bilirubin, alkaline phosphatase, SGOT SGPT and prothrombin time were normal Protein electrophoresis showed an elevation of the α globulin, but immunoelectrophoresis was normal. LE cells were not found and tests for ANA (antinuclear antibody) were negative. Coombs test was negative. Serum iron was 18 μg/100 ml (normal 65–120 g/100 ml) and whole blood folic acid 75 ng/ml (normal 100 ng ml) Bone marrow aspiration revealed a *hypercellular marrow* There were few giant stabs, macroblasts and pronormoblasts, a small number of megaloblasts and a normal number of megakaryocytes, but no budding of platelets was present Prussian blue staining did not reveal any iron deposits. Chest X ray was normal. Because of the serious condition of the patient therapy with blood and platelet transfusions and prednisone 60 mg/day was started. The body temperature remained between 37.5 and 38 C in spite of the therapy with steroids. Repeated blood cultures, chest roentgenograms, intravenous pyelography and X-ray of the gastrointestinal tract were negative The number of platelets increased gradually In October 1972, the platelet count was within normal values, and thereafter it persisted in the normal range. A liver biopsy was normal nd one of the groin lymph nodes revealed a nonspecific histological picture Lymphography performed on in J nuary 1973

eficial and resulted in a gradual rise of the platelet count to normal levels. It is likely that the corticosteroids reduced the sequestration of platelets by reticuloendothelial cells. A retrospective study reported by LACHER [6] revealed that 90 of a group of 402 patients survived more than 10 years after biopsy diagnosis of Hodgkin s disease. 45 of the 90 10-year survi vors required repeated therapy Patients in stages IA and IIA had the best overall 5- (IA 66.2%/ IIA 42.5%/) and 10-year (IA 37.8%/ IIA 36.9%/) survival rate. These patients were treated by orthovoltage radiation therapy using moderate dose without prophylactic therapy Our patient received orthovoltage radiation therapy which resulted in 15 years survival. It is of interest that after MOPP therapy no residual disease was revealed at autopsy Death was due to hepatic necrosis and bronchopneumonia.

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The patient s condition gradually deteriorated, hepatocellular jaundice and coma ensued and the patient died 6 months after operation Postmortem examination demonstrated that death was due to subacute massive liver necrosis and bronchopneumonia. Abdominal lymph nodes showed extensive fibrosis and hyalinization with a small residual number of lymphocytes, plasma cells and macrophages containing hemosiderin. Thoracic lymph nodes showed widely dilated sinuses containing histiocytes and a large number of plasma cells. The bone marrow was cellular and showed an increased number of plasma cells and occasional megakaryocytes. No recognizable residual Hodgkin s disease was found.

Discussion

Cases of long survival in Hodgkin s disease despite inadequate therapy have been described previously [6] Our patient who was treated in 1957 according to the concepts of therapy prevalent at that time, falls into this category Splenomegaly and thrombocytopenic purpura were the presenting manifestations of recurrence and proved to be secondary to Hodgkin s disease Thrombocytopenic purpura is rare in Hodgkin s disease and only a few well-documented cases have been reported [1 3 4 8 9]

Among 134 patients with Hodgkin s disease observed at the Massachusetts General Hospital between 1966 and 1969 there were three in whom Hodgkin s disease presented as ITP [9] These three patients all had involvement of the spleen by Hodgkin s disease when their thrombocytopenic purpura became manifest Additional cases of ITP associated with Hodgkin s disease were reported by JONES [5] in 1973 and ANTONIO and SHERWOOD [1] in 1976 Thrombocytopenic purpura in Hodgkin s disease might be related to therapy with chemotherapeutic or radiotherapeutic agents [7] RUDDERS [8] described a case which illustrated the coexistence of autoimmune thrombocytopenic purpura and autoimmune hemolytic anemia in Hodgkin s disease By performing an opsinization assay for platelet antibodies, he verified the autoimmune basis of the thrombocytopenic purpura. In their series of 100 patients with secondary thrombocytopenic purpura, DOAN *et al* [2] included four cases of secondary thrombocytopenic purpura associated with Hodgkin s disease but no details are available regarding these cases.

Our patient had not received any therapy in the 12 years preceding his admission to the hospital. The spleen was enlarged and the thrombocytopenia may have been the result of hypersplenism However platelet survival studies were unfortunately not carried out. Steroid therapy was ben

Material and Methods

Our experiments were carried out in 3-month-old male mice of the CFLP strain. Two mice were injected with leucine 2 H (New England Nuclear: 0.8–1.0 mCi of total activity) into the tail vein for 60 sec, and after an hour the animals were perfused by Karnovsky [2] fixative solution. Three other mice were treated intraperitoneally with APS for 6 h, and one mouse 30 h, before isotope injection. The livers and kidneys were dissected and immersed in the same perfusion solution for an additional 15 min and then dehydrated and embedded in Durcupan (Fluka) in the usual way.

Semithin sections of 2, 5 and 10 μm were cut and mounted onto glass slides. The slides were immersed in liquid solution of Ilford 14 em Islon, and 3- to 4-μm thick emulsion layer was formed on the section surface. The slides were placed in a refrigerator and developed with Kodak D 19 B developer after 2, 4 and 10 weeks of exposition. The radioautograms were examined and photographed under light and dark field conditions with an Orthoplan microscope.

Results

The effect of APS was examined in 10 mice. A few minutes after administration of APS the platelet count fell below 5% of the initial value, remained at this level for 2–3 days, then by the 6th day it gradually returned to normal. The packed red cell, hemoglobin, reticulocyte, and granulocyte values of the animals did not change in the 6 days.

Six hours after administration of APS, increased isotope incorporation was observed in the cells of the convoluted tubules of the kidney (fig. 3, 4). Even 30 h after treatment, incorporation of the isotope was slightly increased as compared with the controls (fig. 1, 2).

There was no difference in the incorporation of the isotope in the liver of APS-treated or untreated animals.

Discussion

The serum thrombopoietin level is elevated in APS-induced thrombocytopenia as observed in experiments carried out by Schreiner and Levin [12], Nakeff and Roozendaal [9] and Marosi *et al.* [6]. The site of thrombopoietin production, however, is not known. Some data suggest that the kidney may play an important role in the production of thrombopoietin [4, 7, 8].

In the present experiments with APS, the incorporation of isotope leu-

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Incorporation of H^3-Leucine in the Mouse Kidney in Thrombocytopenia

Attempt to Demonstrate Thrombopoietin Production

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Key Words Autoradiography · Kidney · Thrombopoetin

Abstract. Light and dark field autoradiography of semithin sections prepared 6 h after a treatment with APS showed that the incorporation of H^3-leucine into the cells of the convoluted tubules of the kidney was increased in mice. There was no difference in the H^3-leucine incorporation in the liver either in thrombocytopenic or in untreated animals. The increased incorporation of leucine in the kidney in APS-induced thrombocytopenia showed coincidence with increased thrombopoietin production.

Besides the negative results of the experiments of DE GABRIELE and PENINGTON [1], the experiments of KRIZSA [4], McDONALD [7] and McDONALD *et al.* [8] indicated that the kidney may be necessary for thrombopoietin production and that the kidney itself also could produce it.

All investigators are in agreement that thrombopoietin is a protein-like substance [10], but opinions differ as to the details. It has been claimed to be a glycoprotein [5], α-globulin [11], β-globulin [3] and an albumin [13].

In our present series of experiments we studied the degree of incorporation of H^3-leucine in the kidney and the liver in thrombocytopenia induced by antiplatelet serum (APS).

cine in thrombocytopenia was increased in the kidney while no deviation from normal was found in the liver This suggests that the kidney may play a role in the production of thrombopoietin.

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Fig 3 A large number of silver grains (arrows) was observed in the convoluted tubules of APS-treated animals. ³H-leucine was injected 6 h after APS. The section was exposed for 54 days. × 660

Fig 4 The same detail in dark field Incorporation of the radioactive isotope is indicated by arrows. × 660.

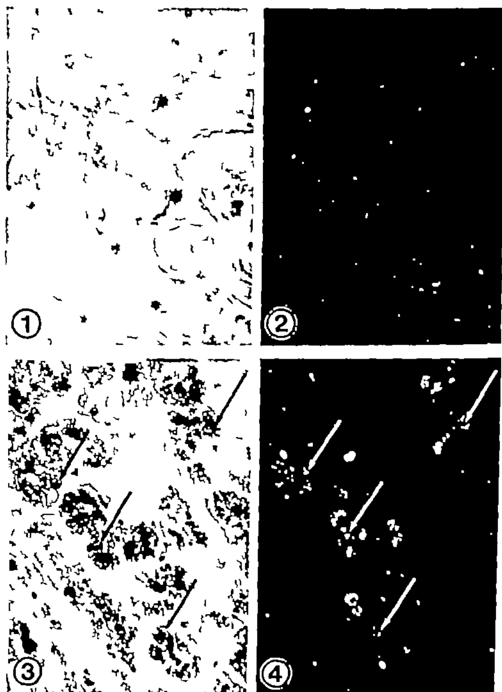


Fig. 1 Detail of the kidney from a control mouse. The section was exposed for 54 days. No specific accumulation of H leucine was detected. × 660.

Fig. The same area as figure 1 in dark field. × 660.

cine in thrombocytopenia was increased in the kidney while no deviation from normal was found in the liver. This suggests that the kidney may play a role in the production of thrombopoietin.

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Fig. 3. A large number of silver grains (arrows) was observed in the convoluted tubules of APS-treated animals. ^{3}H-leucine was injected 6 h after APS. The section was exposed for 54 days. × 660.

Fig. 4. The same detail in dark field. Incorporation of the radioactive isotope is indicated by arrows. × 660.

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Oral Contraceptives, Anti Thrombin III and Fibrinolytic Activity in Africans

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Key Words. Oral contraceptives · Anti-thrombin III · Fibrinolysis

Abstract. A comparison of the effects of progestogen-only and combined progestogen-oestrogen types of oral contraceptives on anti-thrombin III activity and fibrinolysis in African subjects in South Africa is reported. The changes in anti-thrombin III activity are similar to those reported in other races, but the augmentation of fibrinolytic activity is more marked than has been reported elsewhere. The possible significance of these findings is discussed.

Thrombo-embolic disease has been considered to be very rare in the African [15, 16, 22]. On the other hand KALLICHURUM [21] has shown that venous thrombo-embolism is not an uncommon finding at autopsy in Africans dying from a variety of causes in Durban, South Africa. BAKER and HOULDER [4] in a post-operative study in the same hospital population using a ^{125}I fibrinogen technique, found that post-operative DVT was much less common than in white patients undergoing similar types of surgery.

Alteration of the composition of the blood was one of VIRCHOV's [28] triad of aetiological factors of thrombosis, the others being alteration of the vessel wall and blood stasis. Alterations of the blood pre-disposing to thrombosis are usually taken to mean changes in the coagulation and/or fibrinolytic systems.

Recently attention has been focused on the anti thrombin III activity of plasma. Families showing a hereditary deficiency of anti-thrombin III have a higher incidence of thrombo-embolic disease than the general pop-

ulation [11 30 33] VON KAULLA *et al* [29] demonstrated that administration of oral contraceptives containing oestrogen results in a reduction in the anti-thrombin III levels plasma, a result confirmed by several other workers and by alternative techniques [12, 13] The concept that there is normally a balance between coagulation and fibrinolysis was first put forward by ASTRUP [3] and developed by FEARNLEY [14] Enhancement of fibrinolytic activity following the taking of oestrogen-containing oral contraceptive pills has been reported [8, 9] NILSSON and PANDOLFI [24] have suggested that enhanced fibrinolytic activity may constitute a defence against thrombosis.

With increasing sophistication African women are employing contraceptive methods to an increasing extent, including the contraceptive pill, and it seemed of interest therefore to study the effect of oral contraceptives on the fibrinolytic activity and anti-thrombin III levels in African women.

Materials and Methods

The population studied consisted of healthy African women attending family planning clinic who desired or were using oral contraception for non-medical reasons. Oral contraception was offered both with combination oestrogen-progestogen type and with progestogen-only type pills. A group of women attending the clinic for the first time were used as controls. They had never received oral contraceptives and were receiving no other medication. A second group received an oral contraceptive f the combined oestrogen/progestogen type containing progestogen, norgestral 0.5 mg and an oestrogen, ethinyl oestradiol, 0.05 mg: one tablet was taken daily for the first 21 days of the cycle. A third group of subjects received a progestogen-only containing oral contraceptive. This tablet contained norethisterone 0.35 mg: one was taken daily

Serum Anti-Thrombin III Method

Serum anti-thrombin III was measured by the method of VON KAULLA and VON KAULLA [31]. The results are recorded the substrate clotting time in seconds on samples taken at 3, 4, 5 and 6 min. Conventional statistical methods have been employed and the significance determined by Student's t test and the probability (p) assessed from relevant tables.

Fibrinolytic A ty Methods

Tw tests were used to assess fibrinolytic activity:

(1) The euglobulin lysis time This was performed on blood taken by clean venepuncture with minimal stasis using plastic syringes and disposable needles. The blood was mixed with ice-cold 3.8% sodium citrate solution in the proportion of 9

parts of blood to one part of citrate solution in a polythene tube. The tubes were kept in melting ice and the plasma separated in the cold. Fibrinolytic activity is measured as the lysis time of the fibrin clot formed from the euglobulin by a calcium-thrombin solution. Fibrinolytic activity is a direct function of the reciprocal of the lysis time [25]. The results were expressed in arbitrary fibrinolytic units (FU) read from a log/log plot of the lysis time in seconds against arbitrary units, assuming that a lysis time of 10 min equals 100 such units. The units of fibrinolytic activity have been logarithmically transformed to ensure a near-normal distribution in order to meet the requirements of the statistical test

(2) The fibrinolytic activity of the euglobulins prepared as under test 1 were also assessed by the fibrin plate assay Duplicate aliquots of 4 μl were placed on the surface of dry unheated bovine fibrin plates. The plates were incubated at 37 C for 18 h The area of lysed fibrin was measured by determining the product of two perpendicular axes, and expressed in square millimetres, the mean of the duplicates was recorded.

Results

There was a highly significant reduction in the level of anti thrombin III activity in the group receiving the combination type oral contraceptive (table I) Those receiving the progestogen-only pill showed an increase in anti thrombin III levels over the controls which was significant at the 1% level

A highly significant increase in fibrinolytic activity was observed in subjects receiving combination type oral contraceptives but those receiving progestogen-only type showed no significant change from a control group the results of the fibrin plate assay confirmed these findings (table II)

Table I Anti-thrombin III values in subjects on oral contraceptives (substrate clotting time in sec ± SEM)

Incubation period	n	3 min	4 min	5 min	6 min
Group					
Controls	30	17.5±0.9	28.2±1 8	47 4±3 4	75 7±6 9
Ovral	45	14 4±0 5	21 7±1.2	33.2±2.3	52.3±4.5
Micro-novum	41	21±1.3	36.9±2.9	58 3±4 6	95.5±8 4
Comparisons			at 3rd min	at 6th min	
Controls vs. Ovral group			p<0 001	p<0.01	
Controls vs. Micro-novum group			p<0.001	p<0 001	

Table II. Fibrinolytic activity in subjects on oral contraceptives

1 *Euglobulin lysis*

Group		Fibrinolytic activity (FU ± SEM)
Controls		1.05 ± 0.05
Ovral		1.41 ± 0.05
Micro-novum		1.10 ± 0.04
Comparison		
Controls vs. Ovral group	$p<0.001$	
Controls vs. Micro-novum group	$p>0.1$	

2 *Fibrin plate assay*

Group			Area of lysis (mm^2 ± SEM)
Controls			140.4 ± 15.1
Ovral			212.3 ± 14.6
Micro-novum			162.2 ± 11.0
Comparisons			
Controls vs. Ovral group	$p<0.001$		
Controls vs. Micro-novum group	$p>0.1$	<0.05	

Discussion

The association of reduced levels of plasma or serum anti-thrombin III with the taking of oral contraceptives is well documented. However no previous studies specifically in African subjects has been reported to the author's knowledge. In most of the reported studies oestrogen-containing combination type oral contraceptives have been studied and the effect has been attributed to the oestrogen component. Administration of synthetic oestrogen to males in the treatment of prostatic cancer and to menopausal women has been shown to depress anti-thrombin III levels [32]. A few studies have been published on the effect of progestogens on anti-thrombin III. FAGERHOL *et al.* [12], FAGERHOL and ABILDGAARD [13] and HOWIE *et al.* [18] found that there was little change in anti-thrombin III levels. BERGSJÖ *et al.* [5] estimated anti-thrombin III by immunological methods in women receiving chlormadinone and found it to be elevated compared with controls. Our results, showing increased anti-thrombin

parts of blood to one part of citrate solution in a polythene tube. The tubes were kept in melting ice and the plasma separated in the cold. Fibrinolytic activity is measured as the lysis time of the fibrin clot formed from the euglobulin by a calcium-thrombin solution. Fibrinolytic activity is a direct function of the reciprocal of the lysis time [25] The results were expressed in arbitrary fibrinolytic units (FU) read from a log/log plot of the lysis time in seconds against arbitrary units, assuming that a lysis time of 10 min equals 100 such units. The units of fibrinolytic activity have been logarithmically transformed to ensure a near-normal distribution in order to meet the requirements of the statistical test.

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Micro-novum	41	22.1 ± 1 3	36.9 ± 2.9	58 3 ± 4 6	95 5 ± 8 4
Comparisons				at 3rd min	at 6th min
Controls vs. Ovral group				$p < 0.001$	$p < 0.01$
Controls vs. Micro-novum group				$p < 0.001$	$p < 0.001$

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III activity with progestogen-only type oral contraceptives, are similar to those described by BERGSJÖ *et al* [5] Published work on the effect of steroid hormones, including oestrogens and progestogens, on the fibrinolytic activity of blood has shown much variability DUGDALE and MASI [10] have reviewed the published findings. Many workers report no change in fibrinolytic activity following the taking of oestrogen-containing oral contraceptive pills but there are a number of reports of increased fibrinolytic activity [8 9 17] There are few studies of fibrinolytic activity in women receiving progestogen-only type of oral contraceptive HOWIE *et al* [18] report no significant change

The increase in fibrinolytic activity reported here in African women is considerably greater than that reported by other workers in other races. A possible explanation of this may lie in the observation by several groups of workers [7 15 23] that South African blacks normally show a higher level of spontaneous fibrinolytic actvity than do South African whites. The reason for this has never been determined. It has been supposed that this increased spontaneous fibrinolytic activity may be responsible for the reported low incidence of thrombotic disease in South African blacks compared with whites [15]

The increased fibrinolytic activity induced by the combination type pill might be expected to offer protection against thrombosis in accordance with Astrup s theory On the contrary there is an increased incidence of thrombosis in those taking oestrogen-containing contraceptive pills [20 27] A similar situation pertains to fibrinolytic activity and incidence of thrombosis during pregnancy During the latter part of pregnancy fibrinolytic activity is reduced [6 26] but the incidence of venous thrombosis is low [1 2] whereas after parturition there is a rapid rise in fibrinolytic activity and at the same time the incidence of thrombosis, especially deep venous thrombosis of the legs, increased markedly [1 2, 19]

It seems unlikely therefore that enhanced fibrinolysis protects against thrombosis and reasons other than the high spontaneous fibrinolytic activity should be sought for the reported lower incidence of thrombosis in Africans

It is of interest that the progestogen-only type of oral contraceptive did not result in reduction of anti-thrombin III levels. If there is a cause and effect relationship between reduction of anti thrombin III levels and thrombosis, the advantage of using progestogen-only contraception particularly in women with a high risk of thrombosis, e g. in cardiac patients, is obvious.

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Permeability of Membrane to Potassium in Hypochromic Red Cells with Different Specific Density[1]

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Key Words. Iron deficiency anaemia · Red cell potassium · Red cell specific density · Thalassaemia

Abstract. In hypochromic anaemias (heterozygous β-thalassaemia and iron deficiency anaemia) lighter red cells lose more K than heavier ones, following incubation at 37 °C for 24 h. Both in the light and heavy fractions two subpopulations of cells with different permeability to K can be separated by new centrifugation after incubation. On the basis of the results, relationship between K permeability and probability of survival in hypochromic cells is suggested.

It has been known for some time that, on the basis of different specific density red blood cells (RBC) from homozygous [7] and heterozygous [3] β-thalassaemic subjects can be separated by centrifugation into two cellular fractions with different characteristics. (1) A lighter fraction in which the younger cells are concentrated contains the RBC showing a greater derangement of globin synthesis, a larger volume and a higher content of haemoglobin A_2. (2) The heavier fraction contains the older RBC, which have a higher mean corpuscular haemoglobin concentration, a larger content of haemoglobin F and a more marked reduction in their osmotic fragility

In addition, the RBC of subjects with heterozygous β-thalassaemia (Th) show an increase in the permeability of the membrane to potassium (K) which shows itself by cation flux studies [4] and by a consistent loss of cation during the incubation of cells at 37°C for 24 h [10 11] Analo-

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Table I. RBC potassium values (mEq/10[13] cells ±SD) and their percent variations after incubation of whole blood and of light and heavy cellular fractions

	Normals (n=14)	Thalassemics (n=9)	Iron deficients (=8)
Whole blood			
Fresh cells	87.7±9.5	78.6±7.6	88.1±10.3
Incubated cells	78.2±6.6	45.9±16.0	67.0±19.4
Percent decrease	−11	−42	−24
Top fraction			
Fresh cells	98.4±10.5	91.2±10.9	100.5±15.9
Incubated cells	88.5±9.7	45.6±13.8	69.3±15.6
Percent decrease	−10	−50	−31
Bottom fraction			
Fresh cells	79.4±10.3	67.6±7.3	77.7±7.5
Incubated cells[1]	73.2±9.9	48.9±11.3	63.5±8.5
Percent decrease	−8	−28	−18

Incubation in their own plasma at 37°C for 24 h.

obtained by centrifugation, both before and after incubation at 37 C for 24 h.

Unseparated RBC from all three sources lost K on incubation. The loss was most marked for Th cells (−42%), slight in normal cells (−11 /) and intermediate for IDA cells (−24 /). In addition, whereas in normal RBC loss of K was similar for light and heavy fraction, in both types of hypochromic cells the loss of K on incubation was considerably greater in the light (top) fractions (Th:top, −50%, bottom, −28%/ $p<0.001$ IDA. top, −31 / bottom, −18%/ $p<0.005$).

The top fraction of all three types of RBC, when subjected to a second centrifugation after incubation, separated into two subpopulations: a lighter one (top of top incubated) and a heavier one (bottom of top incubated). An analogous separation was obtained with the initial bottom fraction which, on centrifugation after incubation, also gave rise to two subpopulations: a lighter one (top of bottom incubated) and a heavier one (bottom of bottom incubated). The average K contents of these cell fractions are presented in table II their percentage variations were referred to the K content of the corresponding fresh cell fraction. The lighter subpop-

gous behaviour is also seen in hypochromic RBC of subjects with iron deficiency anaemia (IDA) [2, 5]

It is uncertain whether an increase in the permeability of membrane to K can be attributed to the presence of excess globin chains, resulting from an imbalance of globin synthesis, or whether it depends on other factors (e g. the lower haemoglobin content of hypochromic cells, an increase in the ratio surface area/volume of these cells, etc.) NATHAN *et al.* [9] by studying the RBC of subjects with Cooley s anaemia have shown a greater glycolytic activity and K flux in the younger cells which contain a greater quantity of precipitated α-globins. On the other hand, an increase in the permeability to K^+ is also seen in the RBC of subjects doubly heterozygous for α and β-Th, in which globin synthesis seems to be well balanced [6]

In this work we have studied the permeability of the membrane to K^+ of normal and hypochromic RBC of different specific density

Materials and Methods

RBC from 14 normal, 9 heterozygous β-thalassaemic and 8 sideropenic subjects have been separated by the centrifugation technique described by MURPHY [8] The erythrocyte K content was determined for each subject on the following cellular fractions.

(1) On whole RBC both immediately after withdrawal and after incubation at 37 °C for 24 h under sterile conditions in their own plasma. (2) On the light and heavy fraction, obtained by centrifugation of the same cells, before and after incubation at 37 °C for 24 h in their own plasma.

After incubation, the light and heavy fractions were, in several cases (6 normals, 7 heterozygous β-thalassaemics and 4 hypochromic sideropenics), subjected again to the same technique of centrifugation [8].

In each case the K content of the RBC was measured by the following method. the cells were washed three times with 120 mM $MgCl_2$, and then 0.2 ml of packed cells were lysed in 9.8 ml of 0 1% Triton X 100 in distilled water K concentration was measured on each haemolysate by flame photometry and referred to a constant number of 10^{13} RBC, as calculated from mean corpuscular haemoglobin content of the original blood and the haemoglobin concentration of each haemolysate.

Results

Table I shows the average cellular K content of unseparated RBC (whole blood) and of the light (top) and heavy (bottom) cellular fraction

and IDA) is a characteristic present to an equal extent in all hypochromic cells, or whether it is more pronounced in cells with particular characteristics of age, haemoglobin content and manner of synthesis of globin.

The results complement those published by KNOX MACAULAY and WEATHERALL [5] confirming that the increased permeability to K is more pronounced in the lighter fraction of hypochromic cells (in which the younger cells are included) but it is also present to a smaller extent in the whole erythrocyte population.

Since it is probable that cells contained in the same fraction lose different quantities of K and water during the incubation, modifying their specific density it will be possible to separate them by a second centrifugation after incubation at 37 C for 24 h. In fact, the centrifugation of the top fraction after incubation produced two subpopulations of cells: the lighter (top of top incubated) which had a relatively small loss of K in comparison with the same fresh cells the heavier fraction (bottom of top incubated) in which the red cells with a greater permeability to K were concentrated. Since not all of the young cells included in the top fraction have the same probability of survival, one may presume that the first subfraction contains cells which will succeed in ageing, and the second contains the cells which will die more quickly These latter probably occur in a smaller quantity in normal subjects than in subjects with Th or IDA, where an intrinsic cellular abnormality is present.

An analogous subdivision into two subpopulations with varying extent of loss of K was obtained by centrifuging (after incubation) the older RBC (bottom) one may probably infer that amongst the cells that have lived longer some still show optimal functioning of the pump mechanism and have a permeability of the membrane to K near to normal, while others are probably near the end of their life cycle and are succumbing to collapse of the cation pump. In these latter cells the loss of K is greater in hypochromic than in normal RBC, probably because in hypochromic cells the effects of cellular ageing are added to the effects of the intrinsic anomaly of the membrane associated with the hypochromic condition.

From the results the following conclusions may be drawn

(1) A reciprocal relationship seems to exist between K permeability and cell survival. the cells with a greatly altered permeability of the membrane to K are less likely to survive and the ageing of the cells increases their K permeability by means of metabolic exhaustion. Therefore, the loss of K besides being the secondary symptom of a cellular anomaly may increase peripheral haemolytic phenomena, particularly in microcir

Table II Potassium content (mEq/10^{13} cells ± SD) of light and heavy RBC subpopulations, obtained by centrifugation of top and bottom fractions after incubation[1]

	Normals (n = 6)	Thalassaemics (n = 7)	Iron deficients (n = 4)
Top of top			
Incubated[1]	91.1 ± 10.7	61.4 ± 21.2	83.3 ± 11.5
Percent decrease	−7	−33	−7
Bottom of top			
Incubated[1]	73.6 ± 7.4	23.4 ± 10.7	44.6 ± 13.3
Percent decrease	−25	−74	−50
Top of bottom			
Incubated[1]	76.5 ± 5.9	59.9 ± 11.2	70.6 ± 7.6
Percent decrease	−5	−9	−8
Bottom of bottom			
Incubated[1]	6[illegible].5 ± 8.4	29.1 ± 9.4	51.8 ± 13.6
Percent decrease	−23	−56	−33

[1] Incubation in their own plasma at 37 °C for 4 h.

The percent variations are referred to the K^+ content of the corresponding fresh cell fraction.

ulation obtained from centrifugation of the initial top fraction after its incubation (top of top incubated) showed a loss of K^+ content with respect to the fresh top (normal, −7%, Th, −33%, IDA −7%). The heavier subpopulation (bottom of top incubated) lost a greater ($p = 0.02 \div 0.001$) percentage of K^+ in all groups (normal, −25%, Th, −74%, IDA, −50%). With the two subpopulations obtained by centrifugation of the initial bottom fraction after its incubation, the lighter (top of bottom incubated) lost some K^+ in comparison with the fresh bottom in all three types of cells (normal −5%, Th, −9%, IDA, −8%). The heavier subpopulation (bottom of bottom incubated) showed a much higher loss of K^+ in all three groups (normal, −23%, Th −56%, IDA, −33%, $p = 0.05 \div 0.001$).

Discussion

The object of our research was to see if the increased permeability of the membrane to K^+ found in hypochromic cells (from heterozygous β-Th

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culatory areas, where environmental conditions, causing the decline of the active pump mechanism, are similar to those reproduced *in vitro* by incubation at 37 C for 24 h.

(2) Since it is known that in Th the lighter cell fraction contains the cells principally affected by thalassaemic anomaly [3 7] – even if it is not made up exclusively of these – a relationship between imbalance of globin synthesis and increased permeability to K^+ is likely

(3) A similar behaviour of RBC with respect to K^+ permeability between Th and IDA could suggest the existence of a similar anomaly of the membrane in the two types of hypochromic anaemia, even if quantitatively of different degree. This anomaly is not known it could depend on two factors. (a) the unbalanced synthesis of globin chains, which is demonstrated in Th cells but is controversial in IDA ones [1 12] and (b) the reduced haemoglobinization of RBC, which is a property common to Th and IDA.

Studies are in progress to elucidate the degree of responsability of these factors for the abnormal K^+ permeability in hypochromic RBC.

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in European countries, isoimmunisation due to blood group incompatibility has not been found to be an important cause for the haemolysis that leads to severe neonatal jaundice in many Asian countries including West Malaysia. In most cases, the cause for hyperbilirubinaemia is unknown in Asian countries. Because bilirubin is a breakdown product of red blood cells, increased production of bilirubin occurs primarily as a result of increased breakdown of erythrocytes due to haemolysis or bleeding. The neonate s liver is unable to conjugate bilirubin adequately because of a deficiency of the enzyme glucuronyl transferase in the liver cells. This leads to impairment of excretion of bilirubin via the liver rendering the neonate unusually susceptible to hyperbilirubinaemia.

Much attention has recently been focused on disorders of red cell metabolism as a cause of increased haemolysis – especially on the most important metabolic pathway in the red cells, the glycolytic pathway. Deficiency of glucose-6-phosphate dehydrogenase (G6PD), which operates in the oxidative hexose-monophosphate shunt of this metabolic pathway is known to play an important role in severe neonatal jaundice in different Asian countries. In our present study we examine the possible role of several other enzymes important in the defense mechanism of the red blood cells and abnormalities of haemoglobin production in association with severe neonatal jaundice.

Material and Methods

Infants with severe neonatal jaundice were referred to the Paediatric Unit of the General Hospital, Kuala Lumpur Malaysia by other hospitals and by private doctors. The blood of 332 babies (51 premature and 281 full term) with severe neonatal jaundice not due to isoimmunisation or infections were further examined at the Institute for Medical Research, Kuala Lumpur Malaysia. The blood was examined for abnormalities of haemoglobin production and the activity of red cell pyruvate kinase (PK), G6PD glutathione peroxidase (GP), glutathione reductase (GR) and flavine adenine dinucleotide (FAD), stimulation of GR activity and the level of glutathione was estimated. The amount of blood obtained was not always sufficient to carry out all studies but all samples were examined for abnormal haemoglobin and G6PD deficiency

Haemoglobin analysis was carried out by starch-gel electrophoresis using Tris EDTA-boric acid buffer pH 8.6, and discontinuous Tris-boric acid buffer pH 9.5, and cellulose acetate electrophoresis at alkaline pH, and where indicated, agar-gel electrophoresis at pH 6.2 [21]. Sickling test was carried out using metabisulphide as reducing agent. Structural studies were carried out according to techniques described by Lehmann and Huntsman [9].

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Red Cell Metabolism and Severe Neonatal Jaundice in West Malaysia[1]

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Key Words. Red cell metabolism · Neonatal jaundice · Abnormal haemoglobins · G6PD deficiency · Enzyme activities

Abstract. A study was carried out of 332 babies suffering from severe neonatal jaundice who were admitted to the General Hospital, Kuala Lumpur, Malaysia. Of the 332 neonates, 51 were premature and 281 were full-term babies, 178 (110 Chinese, 58 Malay, 9 Indian and 1 European-Pakistani) had bilirubin levels of 20 mg% or higher, requiring exchange blood transfusion. Of the Chinese neonates, 23 (20.9%) had G6PD deficiency, 9 (8.2%) had Hb Bart's and 2 (1.8%) had an abnormal haemoglobin, one Hb Q and one fetal variant. Among the Malay infants, 10 (17.2%) had G6PD deficiency, 7 (12.1%) had Hb Bart's and 10 (17.2%) had abnormal haemoglobins (four had Hb E trait, one had Hb K and Bart's in addition to Hb E, three had Hb CoSp with Hb Bart's, one had Hb Q and one Hb Tak). One of the nine Indian neonates had G6PD deficiency and one had Hb S trait. The one European-Pakistani baby was a carrier of Hb D Punjab. In addition to G6PD deficiency, abnormal haemoglobins seem to have contributed to the high incidence of severe neonatal jaundice in Malaysia. The mean activities of GP, GR and GR after stimulation with FAD were higher, while the mean activity of PK and mean level of reduced glutathione were lower than in normal cord bloods. The percent increase of GR after FAD stimulation was significantly lower; fewer in this group had increases above 20% than in normal cord blood. The possible significance of the findings is discussed.

Severe neonatal jaundice that may lead to kernicterus and often to death is a serious medical problem in West Malaysia. Unlike the finding

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Table I G6PD deficiency and abnormal haemoglobins in severe neonatal jaundice

Race	Number examined	G6PD	Hb Bart's	Abnormal Hb
Chinese	110	23 (20.9%)	9 (8.2%) (77% >5% level)	2 (1.8%) 1 Hb Q 1 Hb F variant
Malay	58	10 (17.2%)	7 (12.1%) (40% >5% level)	10 (17.2%) 4 Hb E 1 HbE + Hb K + Hb Bart's 3 Hb CoSp + Bart's 1 Hb Q 1 Hb Tak
Indian	9	1	0	1 Hb S
European-Pakistani	1	0	0	1 Hb D Punjab
All races	178	34 (19.1%)	16 (9.0%)	14 (7.9%)

Table II Abnormal haemoglobin in the general Malay population and in Malay severe neonatal jaundice patients

	General population						Severe neonatal jaundice		
	adults			newborns					
			%	n		%			%
Our group (1971 1973)	949	Hb E	3.5	1,344	Hb E	1.7	58	Hb E	8.6
		Hb CoSp	2.0		Hb CoSp	1.3		Hb CoSp	5.2
		Hb A$_2$ Ind.	0.5		Hb Q	0.07		Hb Q	1.7
		Hb Q	0.3		Hb D	0.7		Hb Tak	1.7
		Hb D	0.1					Hb K	1.7
		Hb K	0.1						
VELLA [23] (1962)	388	Hb E	1.9						

ity 1.35 1.27 and 1.12 U/10^{10} red blood cells. Two of the babies had one parent with low erythrocyte PK activity and one parent with normal erythrocyte PK activity. The parents of the third baby could not be examined for erythrocyte PK activity.

PK activity was assayed by the method of VALENTINE and TANAKA [22], G6PD by the semiquantitative method of MOTULSKY and CAMPBELL KRAUT [19], GR by the method of BEUTLER [2], GP by the method of PAGLIA and VALENTINE [20] and the level of reduced glutathione was estimated by the method of BEUTLER *et al.* [3].

Haematological examinations followed standard methods. Direct and indirect reacting bilirubin was estimated by the method of MALOY and EVELYN [18]

Results

Bilirubin levels in the 332 infants examined ranged from 16 to 52 mg%. Among the full-term infants, 178 (110 Chinese, 58 Malay 9 Indian and 1 European Pakistani) had bilirubin levels of 20 mg% or higher requiring exchange blood transfusion. Frequencies of G6PD deficiency Hb Bart s and abnormal haemoglobins in this last group are listed in table I.

It should be pointed out that the presence of abnormal haemoglobins in the newborn can easily be overlooked since the amount present at birth is usually much lower than that found in adults, and slow moving haemoglobins tend to overlap with Hb F present in the newborn. In the present study six patients had an abnormal haemoglobin that moved between Hb A and Hb A_2 at alkaline pH one had Hb S one had Hb D Punjab [1] two had Hb Q one had Hb Tak [5] and one had a fetal variant. We routinely studied the parents of these children and found that their abnormal haemoglobin could easily be detected on starch-gel as well as on celluloseacetate electrophoresis, due to the absence of Hb F which tends to obscure the presence of the abnormal haemoglobin. A more detailed report on the structural studies of Hb D Punjab and Hb Tak together with the haematological findings in the jaundiced babies will be published elsewhere.

Even the well-known Hb E can easily be overlooked or misdiagnosed as Hb A_2 in the newborn period due to its low level in cord blood. However on discontinuous starch-gel electrophoresis at pH 9.5 Hb E can easily be detected even at birth because it moves faster with this method than Hb A_2. Since we routinely used the discontinous buffer system at pH 9.5 we could easily detect Hb E at birth. Hb CoSp was never recorded in neonates until LIE INJO [11] reported its presence in an appreciable number of Malaysian newborns.

The mean activities of PK, GP GR, GR activity after FAD stimulation and the glutathione level in the severely jaundiced babies are listed in table III 3 of the 83 Chinese infants had very low erythrocyte PK activ-

Discussion

Of 178 babies with severe neonatal jaundice requiring exchange blood transfusion, 64 had known abnormalities of red cell metabolism. For all racial groups, the incidence of G6PD deficiency in the severely jaundiced patients is significantly higher than in the general population [12]. As is the case in other parts of Southeast Asia, G6PD deficiency plays an important role in the causation of severe neonatal jaundice in Malaysia. The incidence of G6PD deficiency in this series is, however lower than has been reported from Singapore [4].

It is interesting that the incidence of abnormal haemoglobins in severely jaundiced babies is significantly higher than in the general West Malaysian population. Abnormal haemoglobins are very rare in Chinese, yet 2 of the 110 Chinese babies examined were found to have an abnormal haemoglobin. In the Malays, the 17.2% incidence of abnormal haemoglobins is significantly higher than in the general population in the Kuala Lumpur area where the frequency of Hb E is around 2–5%, Hb CoSp around 2% [13] and other abnormal haemoglobins are rare. Comparison of the frequencies in severe neonatal jaundice and in the general population in Malays is shown in table II. Abnormal haemoglobins are also rare in Indians. The only European-Pakistani infant with neonatal jaundice in this study also had abnormal Hb D Punjab. Further the frequencies of Hb Bart's in the severe neonatal jaundice group, especially in Malays, are higher than in the general newborn population [17]. It is possible that abnormal haemoglobin does contribute to the high incidence of neonatal jaundice in this country. It is known that Hb E is an oxidatively unstable haemoglobin [6] and Hb Tak with an elongated β-chain at the C-terminal end is expected to be less stable than normal haemoglobin because β-tryptic peptide XV which consists of tyrosine and histidine only is important in the cooperativity in the haemoglobin molecule. Although the trait condition of certain abnormal haemoglobins does not lead to any problem in adults, it may possibly lead to increased hyperbilirubinaemia in the newborn period, known to be very sensitive toward oxidative stresses. One has also to keep in mind the possibility that other abnormal haemoglobins not detectable by present methods, the so-called silent abnormal haemoglobins, may possibly have had an influence.

The results of the study of erythrocyte enzyme activities and glutathione level are not conclusive, but they reveal several interesting features. The mean activities of GP, GR, and GR after FAD stimulation

Table III Activity of erythrocyte enzymes and level of reduced glutathione in severe neonatal jaundice

	PK $U/10^{10}$ RBC			GP U/g Hb			GR U/g Hb			GR after FAD stimulation U/g Hb			GR after FAD stimulation % increase			Reduced glutathione mg/100 ml RBC		
	n	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD
Neonatal jaundice	142	2.65	0.69	133	5.23	1.00	140	6.31	1.39	127	6.95	1.22	127	12.61	13 97	118	68 78	13 85
Normal cord blood	470	2.84	0.83	561	4.55	0.91	1051	4 76	1.51	866	5 75	1 43	866	25 1	22.4	408	76.94	3 46
	$t=2.48$ $p<0.02$			$t=7\ 60$ $p<0.001$			$t=11.5$ $p<0.001$			$t=8\ 99$ $p<0.001$			$t=6.12$ $p<0\ 001$			$t=10.83$ $p<0.001$		

Discussion

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Neonatal jaundice	142	2.65	0.69	133	5.23	1.00	140	6.31	1.39	127	6.95	1.22	127	12.61	13.97	118	68.78	13.85
Normal cord blood	470	2.84	0.83	561	4.55	0.91	1051	4.76	1.51	866	5.75	1.43	866	25.1	22.4	408	76.94	3.46
		t=2.48 $p<0.02$			t=7.60 $p<0.001$			t=11.5 $p<0.001$			t=8.99 $p<0.001$			t=6.12 $p<0.001$			t=10.83 $p<0.001$	

reason to suspect an unusual marriage pattern among Chinese in Hong Kong to explain this finding. Probably the screening method used by those workers was not sufficiently discriminative. In our earlier report [14] we were unable to determine the exact frequency of erythrocyte PK deficiency in newborns and adults no extremely deficient individuals were found and low values seemed to overlap normal values. In the present report only three infants had PK activities below 1.5 U/10^{10} RBC and none had levels below 1 U/10^{10} RBC. Therefore, the role of PK deficiency in the causation of severe neonatal jaundice in our present series seems by far not as important as reported by Fung *et al.* [7] in Chinese newborns in Hong Kong.

Acknowledgements. We would like to thank colleagues from the Paediatric Unit, General Hospital, Kuala Lumpur Malaysia, for obtaining blood samples from severely jaundiced babies for our studies, and Mr Ambalavanam s/o Solai for his valuable technical help.

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were higher in these subjects than in the cord blood of normal newborns [14] The mean activity of PK and level of glutathione and mean percentage increase of GR after FAD stimulation are, however significantly lower than in cord blood of normal newborns [16]

It is known that GP activity is lower in cord blood of normal newborns than in normal adults and GROSS *et al.* [8] thought that the low activity of GP is the cause of a greater susceptibility of neonatal red blood cells to oxidation It is therefore surprising to see that in this group of severely jaundiced babies, the mean GP activity is higher than in cord blood of normal newborns. Also in an earlier study [15] we found that a group of newborns with lower GR activity in their cord blood developed a higher mean bilirubin curve in the first week of life. The finding of a higher mean GR activity in the blood of the severely jaundiced babies in the present study does not seem to agree with the results of earlier the survey of cord blood. However this discrepancy may be explained by the fact that the lower mean GR and mean GP activities in earlier studies were found in cord blood, whereas in the present study the enzyme activities were estimated in severely jaundiced infants 1–18 days old. Therefore, they are not entirely comparable It can also be argued that in these severely jaundiced several-day-old babies, many older red cells have been destroyed because of increased haemolysis, leaving a relatively younger red cell population with higher GR and GP activity

The red-cell reduced glutathione in our jaundiced babies is significantly lower than in cord blood of normal newborns reported earlier [14] Perhaps this again can be attributed to the fact that these infants were older since adults have lower reduced glutathione levels than newborns [14] Or the lower reduced glutathione levels along with increased glutathione reductase activity may have resulted from the greater use of reduced glutathione which usually accompanies haemolytic conditions.

Three infants in this group had low erythrocyte PK activity although none was below 1 U/10^{10} RBC. FUNG *et al* [7] reported a significant incidence of PK deficiency of 3 4% in the general newborn Chinese population, of whom two-thirds developed neonatal jaundice higher than 15 mg%. Therefore, among a population of jaundiced infants in that area one would expect a much higher incidence than 3 4% PK deficiency However their finding of 16 heterozygotes and 8 homozygotes among 700 newborn babies examined consecutively is unusual If the Chinese population in Hong Kong were in the Hardy Weinberg equilibrium one would expect to find only 1 homozygote to 16 heterozygotes. There is no

reason to suspect an unusual marriage pattern among Chinese in Hong Kong to explain this finding. Probably the screening method used by those workers was not sufficiently discriminative. In our earlier report [14] we were unable to determine the exact frequency of erythrocyte PK deficiency in newborns and adults no extremely deficient individuals were found and low values seemed to overlap normal values. In the present report only three infants had PK activities below 1.5 U/10^{10} RBC and none had levels below 1 U/10^{10} RBC. Therefore, the role of PK deficiency in the causation of severe neonatal jaundice in our present series seems by far not as important as reported by Fung *et al* [7] in Chinese newborns in Hong Kong.

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Proportions of Mouse Erythrocyte Rosette-Forming Lymphocytes, Immunoglobulin-Bearing Cells and E Rosettes in Patients with Lymphoproliferative Diseases

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Key Words. Human lymphocytes · T lymphocytes · B lymphocytes · Mouse rosette · Lymphoproliferative diseases

Abstract. The proportions of mouse erythrocyte rosette-forming (MERF) lymphocytes, immunoglobulin (Ig)-bearing cells and E rosettes were examined in the peripheral blood of patients with lymphoproliferative diseases. The proportion of MERF lymphocytes was considerably increased in chronic lymphocytic leukaemia. The correlation between the proportions of MERF and Ig-bearing cells, characteristic of healthy individuals, is not observed in the lymphoproliferative diseases. It is suggested that in part of the patients with these diseases, the study of the proportion of MERF cells provides more accurate information on the proportion of lymphocytes of bursal origin than does counting of the Ig bearing cells.

Introduction

Lymphocytes forming rosettes with mouse erythrocytes (MERF lymphocytes) were detected in the peripheral blood of healthy individuals by Stathopoulos and Elliott [1974] who also showed that the proportion of this subpopulation is significantly increased in chronic lymphocytic leukaemia patients. The MERF lymphocytes proved to be immunoglobulin (Ig)-bearing cells which do not possess T cell-specific markers, and in healthy individuals there was a correlation between the proportions of Ig-bearing and MERF lymphocytes [Dobozy *et al.*, 1976]. A similar result was obtained by Grieco and Gupta [1975] in a comparison of the proportions of IgMEAC, and mouse erythrocyte-binding receptor-bearing cells. These results suggest that the MERF lymphocytes are B cells.

In the present work a comparison is made of the proportions of MERF lymphocytes, Ig bearing cells and E rosettes (T cells) in the peripheral blood of patients with various lymphoproliferative diseases.

Methods

Patients. Examinations were carried out on the 36 patients listed in table I; these had not participated in therapy at all, or at least not for 3 months. Diagnoses were made on the basis of the usual haematological and histological criteria.

Lymphocytes were isolated from venous blood containing sodium citrate by density gradient centrifugation, as described by BÖYUM [1968].

Sheep erythrocyte rosette-forming (E rosettes) and MERF lymphocytes were determined by methods described earlier [DOBOZY *et al.* 1976].

For the study of the Ig-bearing lymphocytes, monolayers were prepared from the lymphocyte suspension; these were dried in air, and without fixation were stained with FITC-labelled monospecific anti-human antisera directed against the five main heavy-chain classes of Ig [CORMANE *et al.* 1974]. Comparative studies by PAPAMICHAIL [1976] on the differentiation of B and T mouse lymphocytes have proved that the air-dried smear method is equivalent to the cell suspension technique.

Results and Discussion

The results are given in table I. The examinations revealed cases in whom the proportion of Ig-bearing lymphocytes was high and that of the MERF cells was normal or low, and others in whom the proportions of the two markers varied in the reverse manner. In yet other patients the proportions of the cells bearing these two membrane markers were both high or remained in the normal range. The proportion of MERF lymphocytes was primarily increased in patients with chronic lymphocytic leukaemia, and only rarely in other lymphoproliferative diseases. This latter observation supports the findings of STATHOPOULOS and DAVIES [1976].

In healthy individuals significant correlations could be observed between the proportions of MERF lymphocytes and Ig bearing cells [DOBOZY *et al.*, 1976] and the proportions of IgMEAC and mouse erythrocyte binding receptor bearing cells [GRIECO and GUPTA, 1975]. Such correlations could not be detected in the majority of lymphoproliferative diseases. We consider that these diseases, independently of whether they affect primarily the bursa- or the thymus-dependent system, influence the functional state of the B lymphocyte system directly or indirectly in all

Table I Proportions of MERF and Ig-bearing lymphocytes and E rosettes in the peripheral blood of patients with lymphoproliferative diseases

Patient, No.	Diagnosis	MERF cells, %	Ig-bearing cells, %	E rosettes, %
1	CLL	60	55.75	[illegible]
2	CLL	52	9.75	5
3	CLL	73	39.5	20
4	CLL	32	28	48
5	CLL	45	72.5	30
6	CLL	45	29.25	18
7	CLL	57	48.5	11
8	CLL	65		29
9	HL	37	41.75	60
10	HL	51		40
11	HL	11	41	60
12	HL	14	65.75	45
13	RS	13	78.75	47
14	RS	11	20.5	66
15	LS	23	15	75
16	MF	20	11.25	73
17	MF	28	20.75	63
18	MF	15	19.25	51
19	SS	30	19.5	54
20	MM	17	42.5	67
21	MM	27	23	63
22	MM	16	40.25	64
23	MM	18	36.75	63
24	MM	15	19.25	51
25	MM	42	77.5	55
26	BMG	24	27.75	66
27	BMG	32	29.75	59
28	BMG	28	30.75	66
29	BMG	9	25.75	70
30	BMG	26	24.25	64
31	BMG	15	24.75	78
32	BMG	44	16.25	50
33	AGG	6	12.25	88
34	AGG	33	4	70
35	LM	[illegible]	51.5	58
36	LM	41	37	47
Normal values ± SD		18.9 ± 4.4 (= 112)	17.6 ± 5.7 (= 20)	68.4 ± 5.9 (n = 37)

CLL = Chronic lymphocytic leukaemia. HL = Hodgkin lymphoma. RS = reticulosarcoma. LS = lymphosarcoma. MF = mycosis fungoides. SS = Sézary syndrome. MM = myeloma multiplex. BMG = benign monoclonal gammopathy. AGG = agammaglobulinaemia. LM = lymphoma malignum.

In the present work a comparison is made of the proportions of MERF lymphocytes, Ig-bearing cells and E rosettes (T cells) in the peripheral blood of patients with various lymphoproliferative diseases.

Methods

Patients. Examinations were carried out on the 36 patients listed in table I; these had not participated in therapy at all, or at least not for 3 months. Diagnoses were made on the basis of the usual haematological and histological criteria.

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cases, and are at times accompanied by a change in the membrane structure of the lymphocytes. In those cases in which the Ig-bearing lymphocytes were multiplied and the MERF cell proportion at the same time was found to be normal or low, it is also possible that some of these Ig-bearing cells are not true B lymphocytes, but, similarly to the lymphocytotoxin of IgG type observed by GRIFONI *et al.* [1972] in patients with Hodgkin's disease, the immunoglobulins are bound only secondarily to the membrane of these lymphocytes.

In patient No. 34, with agammaglobulinaemia, the proportions of Ig-bearing and MERF lymphocytes were 4 and 33%, respectively. This indicates that in these patients one third of the lymphocytes belong to the bursa-dependent system, but as a consequence of some defect these cells had lost their ability to synthetize immunoglobulin. Similar damage may exist in patient No. 2, with chronic lymphocytic leukaemia, and in patient No. 32, with benign monoclonal gammopathy, in whom the proportion of MERF cells was likewise much higher than that of the Ig-bearing cells.

In agreement with the literature data, the proportion of T lymphocytes in the chronic lymphocytic leukaemia patients was found to be substantially lower than in healthy individuals, and in Hodgkin's disease too a moderate decrease in a proportion of E rosettes was observed, whereas in the other cases it lay within the normal range. The sum of the proportions of Ig-bearing cells and E rosettes exceeded 100% in one-third of the patients examined, while with one exception (patient No. 34) the sum of the proportions of the MERF cells and T lymphocytes was less than 100%. There are several possible explanation for this difference. It may be caused by the proliferation of lymphocytes possessing two different markers, as observed by EDELSON *et al.* [1974] in the Sézary syndrome and by SANDILANDS *et al.* [1974] in a not exactly identified lymphoproliferative disorder, or it is also possible that the T lymphocytes secondarily become cells staining with FITC labelled anti human immunoglobulin. This latter hypothesis suggests that in lymphoproliferative diseases, a study of the MERF lymphocytes may give more precise information on the actual proportion of lymphocytes of bursal origin than can counting of the Ig-bearing cells.

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Atypical B Cell Dyscrasia with Bence-Jones Proteinuria and Intracellular Retention of γ-Chains[1]

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Key Words B cell dyscrasia Bence Jones paraprotein Immunofluorescence

Abstract A case of atypical B cell dyscrasia is described. The patient presented with a history of weakness, bone pain, and bleeding. A K type paraprotein was found in serum and urine. The histology of bone marrow was that of a malignant lymphoma, but no enlargement of peripheral lymphoid organs was detectable Peripheral blood lymphocytes were increased in number and included two populations of immature cells, one lymphoid and the other lymphoplasmocytoid Immunofluorescent staining showed both populations to contain λ and γ chains in their cytoplasm. The clinicopathological heterogeneity suggests a malignant clone of B cells undergoing incomplete maturation with arrest at different stages of the cell cycle.

Introduction

In 1967 Osserman [12] introduced the term plasma cell dyscrasia to designate all pathological and biochemical abnormalities considered to represent unbalanced proliferative disorders of the cells that normally synthesize immunoglobulins. Under this generic designation Osserman encompassed plasma cell myeloma, Waldenström s macroglobulinemia, the heavy chain disease amyloidosis, lichen myxedematosus, the monoclonal gammopathies of unknown significance, and transient plasma cell dyscrasia

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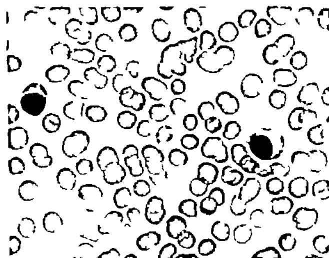


Fig 1 Peripheral blood smear showing two lymphoplasmoblastoid cells. Giemsa. ×450.

Osserman's concept can be broadened to include other situations recently characterized as proliferations of B lymphocytes, such as chronic lymphatic leukemia (CLL) and cases of malignant lymphoma associated with paraproteinemia [1 7 10] CLL has been well characterized in this respect. In most cases, a monoclonal paraprotein can be characterized at the cell surface, sometimes coexisting with intracellularly retained heavy chains or complete paraproteins [5] Furthermore, monoclonal proteins can be detected in a significant proportion of patients with CLL [1 4 7] Indirect evidence for the synthesis of a monoclonal protein by the circulating lymphocytes of a CLL patient was published recently by one of us [16]

Case Report

A 44-year-old Caucasian woman was hospitalized because of progressive dyspnea on exertion. Past history was unremarkable, but the patient had lost 20 pounds over the 2 months prior to admission.

Physical examination revealed thin, white woman in no acute distress. Skin and conjunctiv were pale, with scattered small petechiae on her chest. There was no adenopathy or splenomegaly The liver was palpable 2 cm below the costal mar

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Key Words. B cell dyscrasia · Bence Jones paraprotein · Immunofluorescence

Abstract. A case of atypical B cell dyscrasia is described. The patient presented with a history of weakness, bone pain, and bleeding. A λ-type paraprotein was found in serum and urine. The histology of bone marrow was that of a malignant lymphoma, but no enlargement of peripheral lymphoid organs was detectable. Peripheral blood lymphocytes were increased in number and included two populations of immature cells, one lymphoid and the other lymphoplasmocytoid. Immunofluorescent staining showed both populations to contain λ and γ chains in their cytoplasm. The clinicopathological heterogeneity suggests a malignant clone of B cells undergoing incomplete maturation, with arrest at different stages of the cell cycle.

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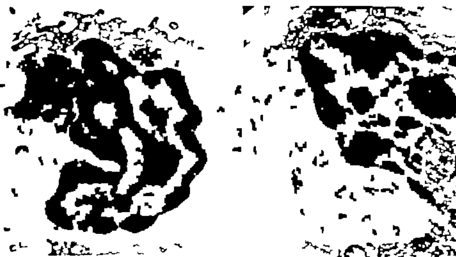


Fig 3 Electron microscopy of peripheral blood lymphocytoid ( ) and plasmoblastoid (b) cells. 25,000.

revealed mild generalized demineralization. Bone marrow obtained from trephine biopsy showed total replacement of marrow elements by cell population characterized by hyperchromatic nuclei with distinct nuclear convolutions and angulations and occasional prominent nucleoli. The cells had the morphologic appearance of lymphoid cells. Mitotic activity was sparse. The morphology suggested poorly differentiated diffuse lymphocytic lymphoma.

Electron microscopy of peripheral lymphoplasmocytoid cells. Tw distinctive cell populations were characterized among the lymphoplasmoblastoid cells of the peripheral blood (fig 3a, b): ( ) immature cells with lymphoid appearance and no endoplasmic reticulum, large Golgi apparatus, several mitochondria, and many free ribosomes with numerous cytoplasmic projections, and (b) immature plasmocytoid cells with lamellar endoplasmic reticulum, several mitochondria, excentric nucleus with prominent nucleolus, large Golgi apparatus, many free ribosomes, electron-dense inclusions, and large pinocytic vacuoles.

Immunofluorescence studies of peripheral blood leukocytes. Peripheral white blood cells were isolated from heparinized blood by sedimentation ith Dextran. Smears were air-dried and fixed in 5% acetic acid 96% ethanol for 15 min at 20 C. They were stained with fluorescein-labeled antisera directed against human albumin, a, and heavy chains, and K and λ light chains. The cytoplasm of numerous cells (10–15% of the white blood cells) was brightly stained by the anti-K antiserum and somewhat less brightly by anti-γ. The morphology of the stained cells appeared lymphocytoid for some, plasmocytoid for others (fig. 4).

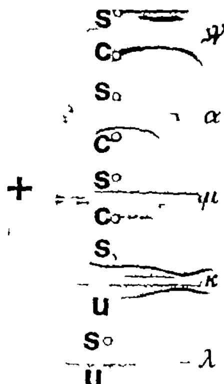


Fig 2 Comparative immunoelectrophoretic study of serum (S) and urine (U) samples from the patient and a normal serum sample used as control (C). Monospecific antisera to IgG (γ), IgA (α), IgM ( ) K and λ chains were used.

gin. Admission laboratory data were ESR 10 mm in 1 h Hb 4 9 g/dl, Htc 17% , reticulocyte count ⁰⁄₀, white blood count 9 600/mm with 4 / metamyelocytes, myelocytes, 6%/ band forms, 23% polymorphonuclear cells, 1 / eosinophils, and 64% lymphocytes, most of which were atypical and suggestive of lymphocytoid plasma cells with occasional small lymphocytes (fig. 1) platelet count 47 000/mm BUN 50 mg/dl creatinine 10.8 mg/dl, creatinine clearance 4 ml min, uric acid 12 mg dl Ca 10.5 mg dl phosphorus 10.1 mg/dl total serum protein 6.9 g/dl

Cellulose acetate electrophoresis showed small homogeneous fractions of mobility Immunoelectrophoresis revealed free λ chains in serum. No bnormal heavy chain was detected (fig 2) Immunoglobulin levels were IgG 3?0 mg dl IgA 9.6 mg/dl and IgM 17 mg/dl Proteinuria was 800 mg/24 h, composed mainly of monoclonal K chains. Sodium dodecyl sulfate-polyacrylamide gel electrophoresi showed that in addition to light chains the patient eliminated considerable amounts of low molecular weight proteins, suggesting that her renal malfunction was due to tubular damage. This was supported by a urinary lysozyme determination of 3.2 mg/?4 h.

Skull X rays showed a few ill-defined lytic areas, films of the lombosacral spine

B cell dyscrasia, with a proliferating population of B cells undergoing some degree of maturation but stopping short of clear differentiation into plasma cells. The finding of B cells in different stages of maturation in the same patient has been previously reported in cases fulfilling the clinical criteria of multiple myeloma [8, 18].

Although immunofluorescence staining of a cell is not a measure of synthetic activity, the fact that the peripheral cells of this patient stained intensely with anti-κ chain antiserum constitutes circumstantial evidence for the cellular origin of the paraprotein. In cases of plasma cell leukemia with Bence-Jones proteinuria [2, 13] peripheral blood plasma cells are thought to participate in the synthesis of the monoclonal protein. In other circumstances, such as in CLL, there is little evidence for or against the synthetic capacity of peripheral lymphocytes. In one reported case, monotypic light chains were identified in lysates of peripheral blood lymphocytes [16], suggesting that the Bence Jones protein present in the urine was synthesized these cells.

Immunofluorescence studies in our patient also showed that the peripheral lymphoplasmoblastoid cells contained γ chains, although no paraprotein containing γ chains could be detected in either serum or urine. Intracellular retention of immunoglobulin heavy chains, free or as part of a complete molecule, has been reported in 'nonsecretory' myeloma, light chain disease, and CLL [5, 6, 9, 11, 14, 15, 17]. This index of poor biochemical differentiation [3] agrees with the hematological evidence of incomplete maturation of a malignant clone of B cells.

Acknowledgements. The authors thank Dr. Mario Ogaw for referring this case to their attention. Mrs. Win Anne Hiff provided excellent technical assistance. We are also indebted to Mrs. Louise B. Just for photographic work and Charles L. Smith for excellent editorial assistance with the manuscript.

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Fig 4 Immunofluorescence study of peripheral blood lymphoplasmoblastoid cells, using a fluorescein-labeled anti-λ chain serum × 470.

The specificity of the fluorescence was established by (a) absorption of the anti λ antiserum with *K* chains and of the anti γ with IgG before staining, and (b) preincubation of the smears with nonfluorescent antisera against λ chains and γ chains prior to incubation with corresponding labeled antiserum In neither case was fluorescence observed. Absorption of the anti antiserum with *K* chains did not decrease the staining of the cells, proving that the staining was not due to cross-reactivity of the anti-γ antiserum with λ chains. Double-staining experiments were not performed.

Discussion

The present case is impossible to classify among any of the currently defined situations associated with paraproteinemia. Bone marrow examination was suggestive of lymphoma but many other features were not, including the absence of splenomegaly and adenopathy and peripheral blood invasion by abnormal lymphoplasmocytic cells. Some features could be considered as close to those of plasma cell leukemia but the degree of bone damage was small and the abnormal cells found in the peripheral blood were of mixed types, some with well-defined characteristics of small lymphocytes. Thus this patient may represent an atypical form of

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Scanning and Transmission Electron Microscopy Study on the Plasma Cells of a Patient with Multiple Myeloma

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Department of Medicine 'B' and Electron Microscopy Unit, Hasharon Hospital, Petah-Tiqva, and Tel-Aviv University Medical School, Tel Aviv

Key Words. Cell ultrastructure · Multiple myeloma · Plasma cells · Scanning electron microscopy · Transmission electron microscopy

Abstract. The ultrastructural features of the plasma cells of a 32-year-old patient suffering from multiple myeloma are described. The high percentage (90%) of plasma cells in the bone marrow aspirate permitted the examination of an almost homogenous population. The appearance of the plasma cells seen with the transmission electron microscope did not differ from that described in other reports. The surface architecture of the plasma cells, such as revealed by the scanning electron microscope, differed from that of the normal and pathological white blood cells. Of particular interest were the membrane-bound portions of the cytoplasm seen as 'buddings' or round bodies in the vicinity of the plasma cells which contained most probably pathological proteins.

The scanning electron microscope (SEM) has been introduced as a useful tool for the study of the surface architecture of blood cells. While the surface features of the normal [1, 2, 9] and leukaemic [3, 4] white blood cells have been reported in detail, the surface appearance of the plasma cells of myeloma patients has not yet been described.

We had the opportunity to examine a patient with multiple myeloma, whose bone marrow contained 90% plasma cells in different maturation stages. The almost homogenous population of plasma cells permitted the examination of their ultrastructure both by transmission (TEM) and scanning electron microscope.

[1] Established Investigator of the Chief Scientist' Bureau, Ministry of Health, Israel.

Case Report

I. K., a 32-year-old Hungarian-born female, was examined because of weakness of 6 months duration. During this period, she developed an increased susceptibility for infections, such as herpes zoster, furunculosis, erysipelas and a few upper respiratory tract infections.

On physical examination she was found in a fairly good general condition, pale. The spleen was palpable 2–3 cm below the costal margin, the liver and the lymph nodes were not palpable. There was a slight tenderness of the skull. Laboratory examinations showed: haemoglobin 11.5 g/100 ml, haematocrit 33%, reticulocytes 0.5%, white blood cells 5,400/μl: polymorphonuclears 38%, band forms 1%, eosinophils 1%, basophils 1%, monocytes 2%, lymphocytes 57%. The platelet count was 135,000/μl. Total protein was 6.3 g/100 ml, albumin 4.2 g/100 ml, globulin 2.1 g/100 ml. Paper electrophoresis of the proteins showed: albumin 56%, α_1-globulin 4%, α_2-globulin 4%, β-globulin 12%, γ-globulin 19% with the presence of an abnormal peak. Immunoelectrophoresis of the globulin showed: IgG 300 mg%, IgA 35 mg%, IgM 35 mg% (normal values 800–1,800, 90–450 and 60–250 mg%, respectively), and two abnormal fractions: monoclonal IgD-lambda, as well as free lambda light chains. Urine examination showed the presence of Bence-Jones lambda protein.

Aspiration sternal bone marrow biopsy revealed that 90% of the cells were plasma cells, in different stages of maturation, frequently seen in groups of 4–6 cells, some of them containing 2–3 nuclei.

Materials and Methods

Bone marrow obtained by sternal aspiration biopsy was immediately transferred into a tube containing cold 1% glutaraldehyde in phosphate buffer. For TEM the cells were postfixed in osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon 812. Thin sections were cut with an LKB Ultratome III and examined with a Philips 300 transmission electron microscope. For SEM the cells were mounted on poly-L-lysine coated glass cover slips [6], dehydrated in graded alcohols and freons and critical point dried with CO_2 using a Polaron critical drying apparatus E 300. Dried specimens were mounted on stubs, coated with gold on a rotary platform and examined with a Philips PSEM 500 scanning electron microscope.

Results

Transmission Electron Microscopy

Most of the cells contained a round, excentric nucleus with a low nucleo-cytoplasmic ratio. The amount of heterochromatin varied, but most of the cells possessed small amounts of condensed chromatin (fig. 1). The

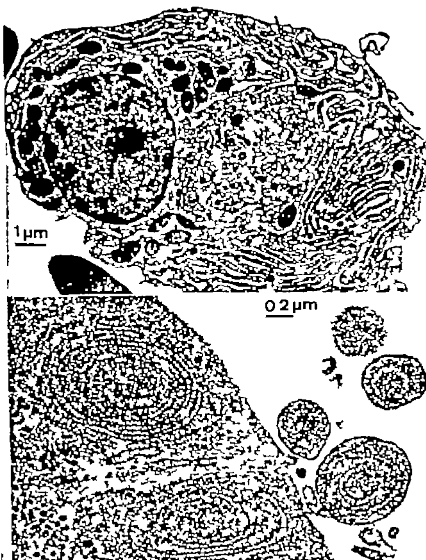


Fig 1 TEM micrograph f patient cell showing low nucleo-cytoplasmic ratio, abundant endoplasmic reticulum and numerous mitochondria surrounding the nucleus. 7,500

Fig 2 Fingerprint-like appearance of the endoplasmic reticulum. Portions of the cytoplasm surrounded by membrane are seen in the vicinity of the cell. × 28,000.

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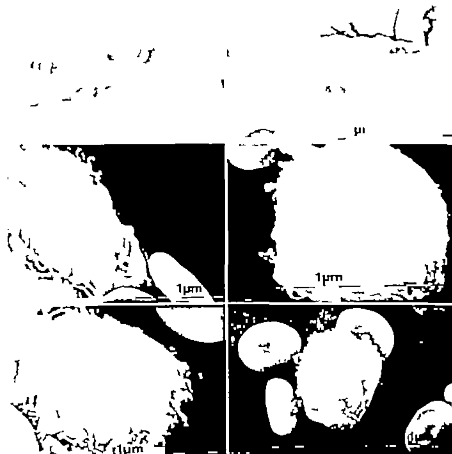


Fig 5 SEM micrograph of round plasma cell Note the round bodies in the vicinity of the cell Original magnification 5,000

Fig 6 SEM micrograph of an oval plasma cell Round bodies are seen in the surrounding red blood cells. Original magnification 5,000.

Fig 7 Higher magnification of plasma cell with numerous villous formations. Original magnification 10,000.

Fig 8. SEM micrograph of plasma cell ith smooth surface. In the periphery of the cell few illous processes can be seen Original magnification × 10,000.

Fig 9 I this plasma cell, processes of different size and appearance are demonstrated Original magnification 10,000

Fig 10 SEM micrograph of plasma cell with "buddings on the cell surface. Original magnification 5,000



Fig 3 A mitochondrion with concentric cristae ×64,200
Fig 4 Two mitochondria showing thickened cristae 64,200.

The appearance of the cells, such as seen with the TEM, supported the diagnosis. The high percentage of the plasma cells in the bone marrow aspirate made it possible to obtain a homogenous population for SEM examination. The results showed that the surface features of the plasma cells differ from those of normal and pathological white blood cells. The variation in number and appearance of the microvilli and ruffles on the cell surface could be the expression of the phase of the cell cycle, as suggested by PORTER *et al.* [5] and SHOHAM and SACHS [7]. Of particular interest are the round bodies seen on the cell surface as well as in its vicinity. These bodies are most probably identical with those observed with the TEM. Since they contained cytoplasmic material, including endoplasmic reticulum, it is conceivable that they posses also pathological proteins which leak through the surrounding membranes. This assumption is supported by the observation that in one case with non-secretory multiple myeloma the cellular buddings could not be detected with the TEM [8].

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Fig. 11 Round bodies, most probably portions of the plasma cell cytoplasm, are seen near and on the red blood cells. Original magnification × 10,000.

cytoplasm showed presence of large amounts of rough endoplasmic reticulum with fingerprint-like appearance in some of the cells. Very often, cytoplasmic material surrounded by a membrane was observed in the vicinity of the cells (fig. 2). The mitochondria were round, small, usually concentrated in groups around the nucleus. Few mitochondria showed alterations, most probably due to degeneration of the cristae (fig. 3). In other cases the cristae were markedly thickened, giving the mitochondrion a spot like appearance (fig. 4). Centrioles and Russell bodies were frequently found.

Scanning Electron Microscopy

The plasma cells were round (fig. 5) or oval (fig. 6). They possessed thin villous formations, the size and number of which varied from cell to cell (fig. 7, 8). Occasionally, sausage like ruffles were seen on their surface (fig. 9). Very often, round buddings loosely connected with the cells (fig. 10) or completely separated from them were observed (fig. 6, 11).

Discussion

The clinical, haematological and morphological features of the plasma cells of our patient are compatible with the diagnosis of multiple myeloma. Although the disease is usually observed in the late decades, its development in younger patients has been reported [1].

The appearance of the cells, such as seen with the TEM, supported the diagnosis. The high percentage of the plasma cells in the bone marrow aspirate made it possible to obtain a homogenous population for SEM examination. The results showed that the surface features of the plasma cells differ from those of normal and pathological white blood cells. The variation in number and appearance of the microvilli and ruffles on the cell surface could be the expression of the phase of the cell cycle, as suggested by PORTER *et al* [5] and SHOHAM and SACHS [7]. Of particular interest are the round bodies seen on the cell surface as well as in its vicinity. These bodies are most probably identical with those observed with the TEM. Since they contained cytoplasmic material, including endoplasmic reticulum, it is conceivable that they posses also pathological proteins which leak through the surrounding membranes. This assumption is supported by the observation that in one case with non-secretory multiple myeloma the cellular 'buddings' could not be detected with the TEM [8].

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Susceptibility to Autoxidation of Lipids of Paroxysmal Nocturnal Hemoglobinuria (PNH)-Like Red Cells

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Key Words. D-Penicillamine · Erythrocytes · Lipid autoxidation · N-Acetyl cysteine · Paroxysmal nocturnal hemoglobinuria

Abstract. The susceptibility to autoxidation of red cell lipids was studied before and after transformation of normal red cells to PNH-like erythrocytes. The transformation was effected by treatment of the red cells with the sulfydryl compounds D-penicillamine (DP) and N-acetyl-L-cysteine (NAC). The autoxidation was induced by incubating the cells with H_2O_2 and was estimated by measuring the generated malonyl dialdehyde. The susceptibility to autoxidation was significantly higher in DP-treated cells, while the opposite was true for NAC-treated cells. However, both DP- and NAC-treated cells showed a similar sensitivity to lysis by acid serum and about the same degree of acetylcholinesterase (AChE) activity decrease, thus indicating that the susceptibility to autoxidation of lipids is not involved in the determination of complement sensitivity or in the AChE activity decrease of the sulfydryl-treated cells. Finally, since, as evidenced from most of the reported cases in the literature, increased susceptibility to autoxidation is a feature of PNH cells, it seems reasonable to suggest that DP-treated cells should be used in preference to NAC-treated cells as a laboratory substitute for PNH cells.

The erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH red cells) show an abnormal susceptibility to lysis by complement, but the nature of their membrane defect has not yet been elucidated [15, 22]. The *in vitro* transformation of normal erythrocytes to PNH-like red cells, effected by exposing the normal cells to different chemical agents, has been a promising approach to the study of the PNH membrane abnormality. Successfully used agents include mainly some proteolytic enzymes [11, 25] and certain sulfydryl compounds, such as 2-aminoethylisothiouronium bromide (AET) [19], reduced glutathione

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Abstract The susceptibility to autoxidation of red cell lipids was studied before and after transformation of normal red cells to PNH-like erythrocytes. The transformation was effected by treatment of the red cells with the sulfydryl compounds D-penicillamine (DP) and N-acetyl-L-cysteine (NAC). The autoxidation was induced by incubating the cells with H_2O and was estimated by measuring the generated malonyl dialdehyde. The susceptibility to autoxidation was significantly higher in DP-treated cells, while the opposite was true for NAC-treated cells. However both DP and NAC-treated cells showed similar sensitivity to lysis by acid serum and about the same degree of acetylcholinesterase (AChE) activity decrease, thus indicating that the susceptibility to autoxidation of lipids is not involved in the determination of complement sensitivity or in the AChE activity decrease of the sulfydryl-treated cells Finally since, as evidenced from most of the reported cases in the literature, increased susceptibility to autoxidation is feature of PNH cells, it seems reasonable to suggest that DP-treated cells should be used in preference to NAC treated cells as laboratory substitute for PNH cells.

The erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH red cells) show an abnormal susceptibility to lysis by complement, but the nature of their membrane defect has not yet been elucidated [15 22] The *in vitro* transformation of normal erythrocytes to PNH-like red cells, effected by exposing the normal cells to different chemical agents, has been a promising approach to the study of the PNH membrane abnormality Successfully used agents include mainly some proteolytic enzymes [11 25] and certain sulfydryl compounds, such as 2-aminoethylisothiouronium bromide (AET) [19] reduced glutathione

(GSH) [9 11 13] N-acetylcysteine (NAC) [4 5] or D-penicillamine (DP) [4 5]

Among the many factors claimed to be involved in the PNH corpuscular defect, rendering the red cell susceptible to damage and lysis, is increased vulnerability of the red cell lipids to autoxidation [13 14 16], but the contribution of this factor has been doubted [18]

The present work, dealing with the study of the susceptibility to autoxidation of membrane lipids of PNH like erythrocytes produced *in vitro* by treatment of normal red cells with DP or NAC, was undertaken since the relevant observations in the literature have been limited to PNH-like cells produced by treatment with GSH [11 13 16] or proteolytic enzymes only [11]

Material and Methods

Venous blood drawn from 20 normal subjects, aged 18–25 years, was collected in tubes containing heparin free from antioxidants and preservatives ('Pularin Evans Medical Ltd, London) Each blood specimen was divided into three samples, of which the first and the second ones were used for experiments with DP and NAC, respectively while the third one served as control. The blood was used on the same day of collection. After centrifugation at 3 000 rpm for 10 min and removal of serum and buffy coat, the red cells were washed three times with isotonic saline. The autoxidation of red cell lipids of the control sample was assayed immediately after the last washing and that of the other two samples after the transformation of the erythrocytes to PNH like red cells.

The transformation of the red cells to PNH like cells was effected by incubating them with alkaline (pH 8) concentrated (10 g/100 ml) solutions of DP or NAC, according to DE SANDRE *et al* [5] The incubation time was 30 min for DP and 15 min for NAC The PNH like cells were tested by the acid-serum test and their acetyl cholinesterase (AChE) activity in comparison to that of the control cells, was also estimated. The acid-serum test was performed as described by DACIE and LEWIS [3] with the following modification Instead of the use of a pool of compatible serums, a recent compatible serum from one individual for every four consecutive red cell samples was used five young volunteers of blood group AB served as the serum donors. The AChE activity was measured by the Michel method [3]

The susceptibility to autoxidation of red cell membrane lipids was estimated by measuring malonyl dialdehyde (MDA) generated after a hour incubation of the normal or the PNH like cells with hydrogen peroxide (H_2O_2) according to the method of STOCKS *et al* [24]. The method is based on the reaction of MDA a secondary breakdown product of autoxidizing lipids – with thiobarbituric acid and includes red cell catalase inhibition effected by the addition of sodium azide to the buffer used for washing or suspending the red cells. Our results are expressed na nomoles MDA per milliliter erythrocytes.

Table I Acid serum lysis, AChE activity decrease and susceptibility to autoxidation of lipids of normal red cells treated with DP or NAC

Cell specimen No.	Acid serum lysis, %			Decrease in AChE activity %		Autoxidation, nmol MDA/ml red cells		
	serum donor	DP treated cells	NAC treated cells	DP treated cells	NAC treated cells	untreated cells	DP treated cells	NAC treated cells
1	A	25	52	32.8	40.3	66	228	60
2	A	40	58	8.1	25.8	104	314	93
3	A	35	45	9.0	9.1	87	298	58
4	A	43	60	9.4	18.8	88	251	40
5	B	10	11	77.7	24.6	70	271	63
6	B	13	10	10.2	11.9	80	318	47
7	B	18	10	36.4	33.8	111	288	60
8	B	16	12	24.6	20.0	68	302	36
9	C	85	86	13.2	20.6	37	231	34
10	C	85	86	14.1	18.2	47	120	30
11	C	26	32	22.8	12.3	57	242	36
12	C	35	64	20.8	26.4	85	254	38
13	D	15	25	9.1	9.1	64	291	40
14	D	11	22	20.9	14.0	53	313	38
15	D	11	15	26.5	17.7	46	325	38
16	D	10	1	11.9	10.2	66	282	34
17	E	31	32	29.[illegible]	35.[illegible]	54	268	42
18	E	23	90	16.5	28.4	36	171	30
19	F	38	60	17.2	33.3	31	292	30
20	E	10	20	1	38.0	91	296	56
Mean value		29.0	37.5	18.5	22.3	67.0	267.7	45.1
±SD		±22.1	±24.7	±8.7	±10.1	±22.6	±51.[illegible]	±15.7

Results

All 20 blood samples after the treatment of the erythrocytes with DP or NAC gave a positi e acid-serum hemolysis test and showed a decrease of their red cell AChE activity (table I). None of the control blood samples presented any detectable hemolysis, while the percent lysis of the PNH-like red cells ranged between 10 and 85 (mean 29) for DP-treated cell and between 10 and 86 (mean 37.5) for NAC treated cells. From table I it is apparent that DP or NAC-treated red cells from different subjects show a significantly different susceptibility to acid lysis by the same

(GSH) [9 11 13] N acetylcysteine (NAC) [4 5] or D-penicillamine (DP) [4 5]

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and the loss of the capacity to induce the PNH lesion in normal erythrocytes after the blockage of the SH groups of the sulfydryl compounds [4] On the other hand the importance of the SH groups for the integrity of normal red cells has been demonstrated by the observation of JACOB and JANDL [12] that inhibition of the erythrocyte SH groups leads to hemolysis. In addition CANELLOS *et al.* [1] found that PNH erythrocytes are more sensitive to the hemolytic effects of SH inhibition than normal erythrocytes and they correlated the PNH membrane defect with an abnormality in the functional activity of membrane SH groups.

The proposed chemical mechanisms by which the sulfydryl compounds act for the transformation of normal erythrocytes to PNH-like cells include the peroxidation of membrane lipid and the split of membrane protein disulfide bonds (S=S-) by reducing them to sulfydryls (SH) or forming mixed disulfides with them [6, 13 23] That lipid peroxidation may be involved for the induction of PNH-like cells by these compounds was suggested by the observations of MENGEL *et al* [14] who found that similarly to red cells from their 14 patients with PNH, PNH-like red cells produced by treatment with GSH showed an increased susceptibility to lysis by H_2O_2 and formed abnormally great quantities of lipid peroxides during incubation with this agent or during exposure to ultraviolet radiation [13 16] Increased susceptibility to autoxidation of red cell lipids was also found by Russian authors [11] both in their 20 cases of PNH and in PNH like cells produced after treatment with proteolytic enzymes or GSH, by STOCKS *et al* [24] in their sole case of PNH, and by us in one case we had the opportunity to study· PANIKER *et al.* [18] however failed to confirm this finding in their 5 PNH patients. In this context, it is noteworthy that the formation of PNH-like cells by treatment with sulfydryl compounds was not inhibited by the absence of oxygen [6, 9] iron or accumulation of H_2O_2 [15] such findings make lipid peroxidation unlikely to be the cause of the PNH-like transformation. MENGEL *et al* [15] consider that the primary biochemical lesion of the PNH cells, which is responsible for their unusual behavior with respect to complement, is an altered SS-SH configuration close to or on the surface of the cell while the increased susceptibility of the cell lipids to autoxidation represents a secondary effect of the primary change. In this respect, SIRCHIA and LEWIS [..] state that although *the fundamental* nature of the PNH defect has not been clarified, an abnormality of membrane proteins would seem to be the primary cause of the defect, while the possibility that cell lipids are in olved secondarily cannot be excluded.

serum and that different normal sera have a different hemolytic activity towards such cells (compare sera A and C with a strong to sera B and D with a weak hemolytic activity) similar in this respect were the findings of other investigators using AET treated red cells [7 21] The great differences in percent acid hemolysis among our different red cell samples after their treatment with DP or NAC must be attributed to the contribution of both the above factors. Interesting in this connection, is the well known fact that different normal sera have also a different hemolytic activity towards erythrocytes from patients with PNH [10 20] and the finding by FERRONE *et al* [7] that the sera that are more active towards PNH cells in the acid-serum test are generally more active also towards AET treated cells and vice versa.

The AChE activity decrease in the PNH like red cells ranged between 8 1 and 36 4% (mean 18 5 ± 8 7%) for DP-treated cells and between 9 1 and 40.3% (mean 22 3 ± 10 1%) for NAC treated cells.

Finally the autoxidation values (in nmol MDA/ml red cells) of the PNH like red cells ranged between 120 and 325 (mean 267 7 ± 51.2) for DP-treated cells and between 30 and 93 (mean 45 1 ± 15 7) for NAC treated cells, while those of the control erythrocytes ranged between 31 and 111 (mean 67 ± 22.6 table I) Thus, the susceptibility to autoxidation of lipids in DP treated cells was significantly higher than in control cells, while the opposite was true for NAC treated cells.

No quantitative correlation among the acid-serum hemolysis degree the decrease of the red cell AChE activity and the susceptibility to autoxidation of the PNH like erythrocytes has been observed (table I) In this regard, it is worthwile to remind that contrary to what happens with PNH cells [17] no relationship between the intensity of acid hemolysis and AChE activity was also observed by FERRONE *et al* [8] in PNH like cells produced by treatment with AET

Discussion

The action of the sulfydryl compounds on normal erythrocytes for their transformation to PNH-like red cells is considered to be du to their sulfydryl (SH) groups. This view is supported by several observations, as the marked decrease of the SH groups in the various sulfydryl solutions during incubation with normal red cells [13] the strong radioactivity of the erythrocyte stroma after incubation of the red cells with $G^{35}SH$ [23]

and the loss of the capacity to induce the PNH lesion in normal erythrocytes after the blockage of the SH groups of the sulfydryl compounds [4] On the other hand the importance of the SH groups for the integrity of normal red cells has been demonstrated by the observation of JACOB and JANDL [12] that inhibition of the erythrocyte SH groups leads to hemolysis. In addition, CANELLOS *et al* [1] found that PNH erythrocytes are more sensitive to the hemolytic effects of SH inhibition than normal erythrocytes and they correlated the PNH membrane defect with an abnormality in the functional activity of membrane SH groups.

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In the present studies, DP treated cells compared to normal cells showed significantly increased susceptibility to autoxidation of lipids, while the opposite was true for NAC treated cells. This different behavior with respect to lipid autoxidation of red cells after their treatment with these sulfydryl compounds should be ascribed to a prooxidant action of the one compound (DP) and to an antioxidant one of the other (NAC). Indeed, the sulfydryl compounds have been found to have completely different effects (either stimulating or inhibiting) on the autoxidation of lipids [2]. However, both DP and NAC treated cells showed a similar sensitivity to lysis by acidified serum and about the same degree of decrease of AChE activity. So the susceptibility to autoxidation of lipids does not apparently play a role in the determination of complement sensitivity of sulfydryl treated red cells, and this is in accordance with what is believed to be true for PNH cells [15, 22]; similarly, it does not seem to be involved in the decrease of AChE activity of sulfydryl treated cells. Finally, since, as evidenced by the great majority of observations in the literature (see above), increased susceptibility to autoxidation *in vitro* is a feature of PNH red cell lipids, it is reasonable to suggest that DP treated rather than NAC treated cells should be used as a laboratory substitute of PNH cells, as they resemble original PNH cells more closely.

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In the present studies, DP treated cells compared to normal cells showed significantly increased susceptibility to autoxidation of lipids, while the opposite was true for NAC treated cells. This different behavior with respect to lipid autoxidation of red cells after their treatment with these sulfydryl compounds should be ascribed to a prooxidant action of the one compound (DP) and to an antioxidant one of the other (NAC). Indeed, the sulfydryl compounds have been found to have completely different effects (either stimulating or inhibiting) on the autoxidation of lipids [2]. However, both DP- and NAC-treated cells showed a similar sensitivity to lysis by acidified serum and about the same degree of decrease of AChE activity. So, the susceptibility to autoxidation of lipids does not apparently play a role in the determination of complement sensitivity of sulfydryl treated red cells, and this is in accordance with what is believed to be true for PNH cells [15, 22]; similarly, it does not seem to be involved in the decrease of AChE activity of sulfydryl treated cells. Finally, since, as evidenced by the great majority of observations in the literature (see above), increased susceptibility to autoxidation *in vitro* is a feature of PNH red cell lipids, it is reasonable to suggest that DP treated rather than NAC-treated cells should be used as a laboratory substitute of PNH cells, as they resemble original PNH cells more closely.

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O. N. ULUTIN and I. R. PEAKE (eds.): Haemophilia. Proceedings of the IXth Congress of the World Federation of Haemophilia, Istanbul, 20–22 August, 1974. Excerpta Medica, Amsterdam 1975. XI + 273 pp. US $ 37.50. ISBN 90-219-0291-5.

Under the title 'Haemophilia' ULUTIN and PEAKE publish the Proceedings of the IXth Congress of the World Federation of Haemophilia in Istanbul held on the 20th–22nd of August, 1974. The first chapter is reserved to reviews on factor VIII function and modern aspects of haemophilia treatment. Each one of the seven other chapters reports on a special subject. Chapter IV concerning von Willebrand' disease, is probably the most important one which conveys to the reader very valuable information on the possible variants of Willebrand' disease, detected by various and interesting methods. The detection methods are also separately discussed in relationship to diagnosis with reference to variants, well as on the behaviour of factor VIII activity fter transfusion. Chapter VII is devoted to the treatment of haemophilia A patients with inhibitors against factor VIII. Especially interesting is the inquiry made by K. DORMANDY among clinicians with experience in the treatment with immunosuppressive drugs. Her report contains otherwise unpublished data. The more frequently used drug cyclophosphamide appears to have suppressed, in some cases, the immune response characterized by an absence of antibody rise in 62 bleeding episodes in 18 patients out of 45 cases. However it is not possible to discriminate the patients who will benefit from those who will not, nor to recommend regime better than the other ones. Other chapters deal with the detection of female carriers in haemophilia A, possible in about 70% f the cases, on the incidence of haemophilia and on clinical aspects such as treatment of joint bleeding, the estimation and ecovery f factor VIII in fractions and after transfusion and incidence of Australia antigen and antibodies. The experiences made with home treatment and rehabilitation in different haemophilia centers are reported in the last chapter. Reading f 'Haemophilia' the Proceeding of the Istanbul congress, can be highly recommended. F. DUCKERT *Basel*

J. FORRAI: Radiology of Haemophilic Arthropathies, vol. 1 Haematologica. Akadémiai Kiadó, Budapest 1976. 135 pp., US $ 8.50. ISBN 963-05-1012.

Gegenstand dieser Abhandlung ist vor allem die röntgenologische Morphologie des Blutergelenks. Die Rollen der Gerinnungsfaktoren und der Genetik werden dagegen in diesem Rahmen nicht berücksichtigt. Das Büchlein enthält 38 Seiten eines knappen, übersichtlichen Textes, 158 Literaturzitate und einen Atlasteil mit Röntgenbildern von 7? Gelenkuntersuchungen, zum Teil in mehreren Ebenen und mit Zusatzaufnahmen wie Tomographien.

Der Autor berichtet über seine 10jährige Erfahrung als Leiter der Röntgenabteilung am National Institute of Haematology and Blood Transfusion in Budapest. In dieser Zeit wurden 200 Patienten mit Hämophilie betreut, in 97 Fällen erfolgte

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Granulozytenersatzes beim leukopenischen, infizierten Patienten stellt sich heute in jedem medizinischen Zentrum. Für diejenigen, welche diese Möglichkeit bereits besitzen, gibt das Buch wertvolle Hinweise über eventuell methodische und technische Verbesserungen, die angebracht werden könnten. Zentren, die vorhaben, mit dem Granulozytenersatz zu beginnen, gibt es einen guten Überblick über das, was bisher auf diesem Sektor geleistet wurde, und hilft bei der Auswahl der Technik, die sich für die jeweiligen Anforderungen am besten eignet. B. SPECK, *Basel*

Congenital Disorders of Erythropoiesis. Ciba Foundation Symposium 37 (new series). Elsevier/Excerpta Medica/North-Holland, Amsterdam 1976. 408 pp., US $ 27.50. ISBN 90-219-4041-8.

The symposium was held in London, March 12–14, 1975 under the chairmanship of D. J. WEATHERALL. The proceedings contain reviews and discussions of high standard on embryonic erythropoiesis, kinetics and regulation of fetal and adult erythropoiesis, oxygen dissociation studies, pathophysiology, clinical aspects and management of Diamond-Blackfan and Fanconi anaemia, of the congenital dyserythropoietic anaemias and thalassaemias and on the regulation of globin gene expression. The reader is impressed by the rapidly increasing knowledge about disordered erythropoiesis in childhood especially at the molecular level. The book is extremely valuable source of information for the haematologist in clinical practice as well as for the laboratory investigator. H. R. MARTI, *Aarau*

ERNEST BEUTLER. **Red Cell Metabolism.** A Manual of Biochemical Methods, Vol. 2. Grune & Stratton, New York 1975. 160 pp. US $ 14.75. ISBN 0-8089-0861-8.

The first edition of this manual, published in 1971, gained world-wide reputation. In the new edition several prodecures are revised leading to improvements of *variety of methods, e.g. removal of leukocytes and platelets by filtration*, preparation of haemolyzates, new standardized techniques for phosphofructokinase, diphosphoglyceromutase, pyruvic kinase, catalase and acetylcholinesterase, estimation of glycolytic intermediates. Some calculations of enzyme activities have been simplified. The manual is easy to use and represents an essential help for haematologists and biochemists in laboratory practice. H. R. MARTI, *Aarau*

M. J. HOBART and I. MCCONNELL. *The Immune System.* A Course on the Molecular and Cellular Basis of Immunity. Blackwell, Oxford 1975. 358 pp. £ 5.00. ISBN 0-632-00157-7.

This book is based on the Advanced Course in Immunology held annually at the Royal Postgraduate Medical School, Hammersmith Hospital, London. Many distinguished angloaxon immunologists have participated in its elaboration. I four sections, the volume covers immunochemistry, immunobiology, immunogenetics and clinical aspects. No recent book on immunology is so complete, up t date and concise. The print is very pleasant, the graphs are clear and easy to under

eine detaillierte Röntgenanalyse. Wertvoll sind die statistischen Angaben über 77 eigene Patienten mit Hämophilie. Im Textteil wird weiter auf das akute Blutergelenk, die Panarthropathie und schliesslich auf regrediente Veränderungen mit klinischen, pathologischen und röntgenmorphologischen Gesichtspunkten eingegangen. Die Veränderungen an den einzelnen Gelenken werden kurz beschrieben, die Differentialdiagnose abschliessend erwähnt, aber nicht ausführlich erläutert. Literaturzitate, vor allem aus dem anglo-amerikanischen, deutschen und ungarischen Schrifttum, vereinzelt auch aus der französischen und russischen Literatur erfassen vor allem den Zeitraum von 1971 bis 1973 vereinzelt beziehen sie sich auch auf das 19 Jahrhundert

Der Hauptwert dieser Schrift liegt im Atlasteil, der ein ausführliches röntgenmorphologisches Material verschiedener Stadien wiedergibt Leider lässt die Güte der Bildwiedergabe vor allem bei den Knochenfeinstrukturen, einige Wünsche offen. Die wesentliche Bildinformation reicht aber für die Orientierung aus.

Als Hauptaufgabe der Röntgendiagnostik sieht der Verfasser 1 Aufnahmen über den Zustand der Gelenke als Standortbestimmung und 2. spezielle Untersuchungsmethoden Ob man bei der röntgenologischen Abklärung von Kindern unter 6 Jahren bei typischen klinischen Symptomen nur das affizierte Gelenk untersuchen soll – wie der Autor empfehlt – oder doch eine Vergleichsaufnahme anfertigt, ist zumindest diskutierbar

Gewünscht hätte man sich ein Eingehen auf den Wert der Knochenszintigraphie, vor allem in frühen Stadien Der Bildteil könnte ausserdem noch gewinnen durch eine Gegenüberstellung mit den differentialdiagnostisch wichtigsten Knochenveränderungen. Vor allem der ausführliche Bildteil gibt dem kleinen Band seinen Wert für die Beurteilung röntgenmorphologischer Veränderungen bei der Hämophilie. Er ist Radiologen, Hämatologen, Rheumatologen und Orthopäden, die mit diesem Problem konfrontiert werden, zu empfehlen M ELKE

J M GOLDMAN and R. M LOWENTHAL (eds) **Leucocytes: Separation, Collection and Transfusion.** Academic Press, London 1975 XXXVI + 604 pp £ 13.80/ US $ 35 75 ISBN 0-12 288550-3

Dieses Werk gibt einen ausgezeichneten Überblick über den derzeitigen Stand des Granulozytenersatzes bei leukopenischen, infizierten Patienten. Die technischen Aspekte der verschiedenen Methoden der Granulozytenprodukt on nämlich Continuous Flow Centrifugation (CFC) und Filtrationsleukopherese (FL) sowie «des Haemonetic Cell Processors» werden ausführlich dargestellt. Der Wert von Granulozytentransfusionen wird anhand von experimentellen und klinischen Studien dokumentiert. CFC und FL-Granulozyten werden *in vitro* und *in vivo* auf ihre Funktionstüchtigkeit untersucht, und auf Vor und Nachteile der beiden Methoden wird eingegangen. Ein weiteres Kapitel widmet sich dem Einfluss der HL A-Antigene auf das zu erwartende Transfusionsresultat. Spezielle Kapitel sind der Gewinnung hämopoetischer Vorläuferzellen sowie leukämischer Zellen für direkte therapeutische Zwecke wie auch für die Immunotherapie akuter Leukämien gewidmet Ebenfalls eingegangen wird auf den Plasmaaustausch mit der CFC bei Patienten mit paraproteinämischen Erkrankungen, akutem Leberversagen usw Die Frage des

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Results of Three Years' Experience with the Deoxyuridine Suppression Test

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Key Words. Alcohol-induced macrocytosis · Deoxyuridine suppression test · Megaloblastic erythropoiesis · Normoblastic erythropoiesis · Red cell folate · Serum vitamin B_{12}

Abstract. The results of deoxyuridine (dU) suppression tests performed on 400 marrow samples aspirated over 3-year period are summarised. High dU-suppressed values were found in all patients with vitamin B or folate deficiency, in some patients receiving inhibitors of dihydrofolate reductase, in 4 of 19 epileptics receiving anticonvulsants and in 2 of 21 patients with iron deficiency anaemia. High dU-suppressed values were found even in those vitamin B or folate-deficient patients who had serum vitamin B and red cell folate levels within the normal range. Conversely, some patients with subnormal serum vitamin B_{12} levels (including 1 on high doses of penicillin, 1 on anticonvulsant therapy and 2 vegans) and some patients with subnormal red cell folate levels gave normal dU-suppressed values, and were considered not to suffer from the metabolic consequences of vitamin B or folate deficiency. All of the patients with megaloblastic erythropoiesis induced by the antipurine drugs or cyclophosphamide, 4 patients with megaloblastic erythropoiesis associated with primary acquired sideroblastic anaemia, chronic myeloproliferative disorder or subacute erythraemic myelosis and proportion of the patients with megaloblastic erythropoiesis associated with anticonvulsant therapy, chronic alcoholism, acute myeloid leukaemia or myelomatosis also gave normal dU-suppressed values. The addition of 1 µg cyanocobalamin per millilitre of marrow culture together with the deoxyuridine caused significant reduction in the dU-suppressed value in 89% of vitamin B_{12}-deficient patients and the addition of 10 µg pteroylglutamic acid per millilitre of marrow culture caused significant reduction in 91% of folate-deficient and 57% of vitamin B_{12}-deficient patients.

The deoxyuridine suppression test is a recently-introduced method of diagnosing vitamin B_{12} or folate deficiency. This test is based on the observations that the incorporation of ^{3}H-thymidine (^{3}H-TdR) into the DNA of normal bone marrow cells is severely depressed when these cells

stand, and some of the illustrations, e.g., those of antigen-antibody complexes, are just beautiful. The presentation of the subjects has been biased towards the more rapidly progressing aspects of immunology The chapters on immunogenetics, a fast-moving field with a difficult and changing nomenclature, are a good example: complete easy to read and clear: the more technical terms are defined and explained when introduced.

The present book, destined originally for advanced students with particular interests in immunology can equally be recommended to immunologists who have specialized in subfields, as well as to anyone with an interest in immunology who likes a well-written book.

T. L. VISCHER *Geneva*

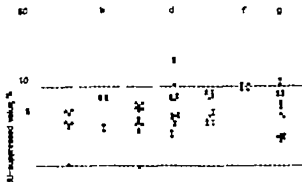


Fig. 1. Deoxyuridine-suppressed values in some of the groups of subjects studied. — Haematologically normal; b — renal failure (O) and chronic liver disease (■)· — subacute and chronic infections (□) and collagen diseases (▲); d — carcinoma, lymphoma and myelomatosis; — acute myeloid leukaemia and subacute erythraemic myelosis (△), chronic lymphocytic and chronic myeloid leukaemia (▽) and the chronic myeloproliferative disorders (▼); f — haemolytic anaemia (untreated with folic acid)· g — iron deficiency The interrupted lines indicate the 95% confidence limits for haematologically normal subjects.

subjects varied between 2.1 and 9.0% [9] With improvements in our radioactive counting technique, the range observed in haematologically normal subjects decreased slightly to 1.4–7.7% with 95% confidence limits of 1.4–8.6% (geometric mean, 3.5%).

Uncomplicated vitamin B_{12} or folate deficiency Marrow aspirates from 33 patients with folate deficiency unassociated with iron deficiency chronic disorders, haemolytic anaemia or chronic alcoholism and 45 patients with vitamin B_{12} deficiency unaccompanied by iron deficiency were studied. In the above patients, the diagnosis of vitamin B_{12} or folate deficiency was based both on clinical features and on the results of various laboratory investigations such as the assay of serum vitamin B_{12} and red cell folate levels, Schilling tests, and other tests of malabsorption. 39 of the 45 vitamin B_{12}-deficient patients suffered from pernicious anaemia and the remaining 6 cases from vitamin B_{12} malabsorption complicating previous gastric surgery All but one of the folate-deficient patients suffered either from a dietary folate deficiency (23 cases) or from coeliac disease (9 cases). In the remaining case, the folate deficiency appeared to be a complication of a previous partial gastrectomy The dU-suppressed values in all 33 patients with folate deficiency and all 45 patients with

are pre-incubated with an appropriate concentration of deoxyuridine [4] and that marrow cells from patients with vitamin B_{12} or folate deficiency show a subnormal suppression of ^{3}H TdR incorporation after pre-incubation with deoxyuridine [4, 5]. In an earlier paper, we [9] described a simple method of performing the deoxyuridine suppression test in which the technique employed for measuring the incorporation of ^{3}H TdR into DNA took at least 6 h less than that employed by other workers [2, 5]. This rapid deoxyuridine suppression test has been in regular use in our department over the past 3 years and has made it possible to aspirate a sample of marrow in the morning and establish the diagnosis of vitamin B_{12} or folate deficiency on the afternoon of the same day, if necessary. The present paper gives a summary of our experience with this test and illustrates its diagnostic value. The results presented confirm and extend observations already published by ourselves and others [1, 3, 8–10, 13] and also include new observations, particularly with respect to the use of the test to distinguish between vitamin B_{12} and folate deficiency.

Materials and Methods

400 patients with a wide variety of diseases were studied. Blood counts were performed on a Coulter Counter, Model S, standardised with 4C Coulter Counter cell control. Serum vitamin B_{12} and red cell folate levels were assayed microbiologically using *Lactobacillus leichmanii* and *Lactobacillus casei*, respectively. In our laboratory, the normal range for the MCV is taken as 82.0–97.1 fl and in the case of the microbiological assays, the lower limits for the normal range are taken as 150 ng/l for the serum vitamin B_{12} level and 110 µg/l for the red cell folate level.

Deoxyuridine suppression tests were performed on bone marrow cells using the technique described by WICKRAMASINGHE and LONGLAND [9]. The incorporation of ^{3}H TdR after pre-incubation with deoxyuridine was expressed as a percentage of the incorporation without pre-incubation with deoxyuridine, this percentage being called the deoxyuridine (dU)-suppressed value.

The effects of adding 10 or 50 µg pteroylglutamic acid (PGA) or 1 µg cyanocobalamin/ml of marrow culture together with the deoxyuridine on the dU-suppressed value were studied in some of the vitamin B_{12}- or folate-deficient patients to determine the value of such data in distinguishing between these deficiency states [11].

Results

dU-Suppressed Values in Various Groups of Patients

Haematologically normal subjects. In an earlier paper, we reported that the dU-suppressed values encountered in haematologically normal

Table I. Summary of the data relating to the 4 epileptics who gave high dU-suppressed values

Case	Drugs	Hb g/dl	MCV fl	Serum B ng/l	Red cell folate µg/l	dU-suppressed values, % without added PGA or B_{12}	with 10 µg PGA/ml	with 50 µg PGA/ml	with 1 µg B_{12}/ml
MB (a)	DPH, Meth	4.7	106	295	98	14.4	9.8	5.5	
MB (b)		7.0	78			8.1			
SR	DPH, Ph	10.0	72	350	153	11.4	11.6	6.4	10.6
CM (a)	Ph, M, S	12.9	102	160	31	17.5	17.5		18.4
CM (b)*		4.6	107	65	130	36.7	17.1	13.4	37.1
CM (c)*		8.6	94	80	133	4.1	2.7		
JD	DPH, M, C	9.6	93	165	164	19.0	8.9	6.3	

DPH = Diphenylhydantoin Ph = phenobarbitone Meth = methsuximide M = mysoline (primidone) S = sulthiame C = carbamazepine

Investigations repeated after taking an improved diet for 1 month.

Investigations repeated 1 year later without change of therapy

Investigations repeated after therapy with 5 mg folic acid daily for 3 weeks.

chronic lymphocytic leukaemia and 1 patient with myelofibrosis gave abnormally high dU-suppressed values (fig. 1c–e). All but one of the patients with a high dU-suppressed value showed megaloblastic erythropoiesis and had red cell folate levels of less than 110 µg/l the remaining patient (with disseminated carcinoma of the kidney and a very poor diet) showed normoblastic erythropoiesis and had a red cell folate level of 270 µg/l. No correlation was found between the logarithm of the red cell folate level and the logarithm of the dU-suppressed value in a group of 25 patients with the anaemia of chronic disorders and a normal dU-suppressed value ($r = -0.13$ $p > 0.1$).

Haemolytic anaemia. Four of the 10 patients with haemolytic anaemia gave abnormally high dU-suppressed values (fig. 1) All 4 of these patients (2 with autoimmune haemolytic anaemia, 1 with hereditary spherocytosis and 1 with dapsone-induced haemolytic anaemia) showed megaloblastic erythropoiesis despite normal red cell folate levels of 147–

viatmin B_{12} deficiency were higher than 9 0%. Statistically significant inverse correlations were found between the dU-suppressed value and the red cell count in both the vitamin B_{12}-deficient ($r = -0.52$ $p < 0.001$) and folate-deficient ($r = -0.61$ $p < 0.001$) groups.

Although all the cases of vitamin B_{12} or folate deficiency mentioned in the preceding paragraph had high dU-suppressed values, 6 of the patients with pernicious anaemia had serum B_{12} levels within the normal range (with values of 165 167 240 328 370 and 550 ng/l) and 5 of the patients with a folate deficiency had red cell folate levels within the normal range (with values of 112, 150 217 224 and 250 μg/l)

The dU-suppressed value obtained after the addition of 10 μg PGA/ml of marrow culture together with the deoxyuridine was at least 15% lower than the dU-suppressed value obtained without the addition of PGA in 30 out of the 33 folate-deficient patients and 20 out of 35 vitamin B_{12}-deficient patients. Those folate-deficient marrow samples which showed little or no reduction of the dU-suppressed value in the presence of 10 μg PGA/ml of culture showed a reduction of 15% or more in the presence of 50 μg PGA/ml of culture.

The addition of 1 μg cyanocobalamin/ml of marrow culture together with the deoxyuridine caused a reduction of 15% or more in the abnormally high dU-suppressed values in 37 of 43 vitamin B_{12}-deficient patients. This concentration of cyanocobalamin also caused a reduction of 15% or more in the dU-suppressed values in 4 of 33 folate-deficient patients. All 4 of these patients were on a very inadequate diet and one of them had a low serum vitamin B_{12} level of 125 ng/l

A statistically significant inverse correlation was found between the red cell folate level and the percentage reduction in the dU-suppressed value induced by the addition of 10 μg PGA/ml of marrow culture in the folate-deficient patients ($r = -0.56$ $p < 0.01$) but not in the vitamin B_{12} deficient group) or 10 μg PGA ($r = -0.14$ $p > 0.1$ for the vitamin B_{12} te-deficient groups, no correlation was found between the serum vitamin B_{12} level and the percentage reduction in the dU-suppressed value induced by the addition of either 1 μg cyanocobalamin ($r = -0.01$ $p > 0.1$ for the vitamin B_{12}-deficient group and $r = -0.34$ $p > 0.05$ for the folate-deficient group) or 10 μg PGA ($r = -0.14$ $p > 0.1$ for the vitamin B_{12}-deficient group and $r = 0.02$ $p > 0.1$ for the folate-deficient group) per millilitre of marrow culture.

Anaemia of chronic disorders Nine of these patients, including 3 patients with carcinomatosis, 2 patients with myelomatosis, 1 patient with

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Case	Drugs	Hb g/dl	MCV fl	Serum B_{12} ng/l	Red cell *folate* µg/l	dU-suppressed values, %			
						without added PGA or B_{12}	with *10 µg* PGA/ml	with *50 µg* PGA/ml	with *1 µg* B_{12}/ml
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CM (b)		4.6	107	65	130	36.7	17.1	13.4	37.1
CM (c)[3]		8.6	94	80	133	4.1	2.7		
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Haemolytic anaemia. Four of the 10 patients with haemolytic anaemia gave abnormally high dU-suppressed values (fig. 1). All 4 of these patients (2 with autoimmune haemolytic anaemia, 1 with hereditary spherocytosis and 1 with dapsone-induced haemolytic anaemia) showed megaloblastic erythropoiesis despite normal red cell folate levels of 147–

Table II Cases with normoblastic erythropoiesis in which the marrow cells gave abnormally high dU-suppressed values

Case	Hb g/dl	MCV fl	Serum B_{12} ng/l	Red cell folate μg/l	dU-suppressed value, %	Diagnosis
DK	3 9	74	165	610	27 1	pernicious anaemia, iron-deficient
SW	11 0	95	87		22.5	pernicious anaemia, hypothyroid
JM	12.8	91	145	1.5	19 1	pernicious anaemia
TJ	9 8	105	710	90	25.2	folate and iron-deficient, ? dietary
CB	10.7	76	168	79	19 4	coeliac disease, folate- and iron-deficient
CE	11.5	85	230	47	14.2	folate-deficient, dietary
EJ	7.0	58	440	719	10.0	iron-deficient
DH	9 4	64	125	342	9 1	iron-deficient

860 μg/l. The addition of 10 μg PGA/ml of marrow culture together with the deoxyuridine caused a reduction of 15% or more in the dU-suppressed value in the cases with hereditary spherocytosis and dapsone-induced haemolytic anaemia but not in the other 2 cases. The bone marrow cells of the 2 patients with autoimmune haemolytic anaemia (with red cell folate levels of 220 and 860 μg/l) only showed a similar degree of reduction in the dU-suppressed value after the addition of 30–50 μg PGA/ml of marrow culture. In the patients with hereditary spherocytosis and dapsone induced haemolytic anaemia, the dU-suppressed value returned to normal and haemopoiesis became normoblastic after therapy with 5 mg folic acid daily for 3 days.

Iron deficiency 21 patients with untreated iron deficiency (Hb 2.7–12.7 g/dl MCV 57–80 fl) were studied (fig. 1) and 2 of them (with chronic gastro-intestinal blood loss) gave abnormally high dU-suppressed values (table II) Both these patients took an adequate diet, gave normal results with the Schilling test and responded completely to oral iron therapy without developing macrocytosis.

Table III. Cases with megaloblastic erythropoiesis in which the marrow cells gave normal dU-suppressed values

Diagnosis	Cases	Hb g/dl	MCV fl	dU-suppressed values, %
Therapy with cyclophosphamide	1	10.3	87	3.5
Therapy with antipurines	20	11.2–17.9	96–125	1.6–7.4
Therapy with azathioprine and cyclophosphamide	4	6.2–15.4	94–129	2.1–7.8
Therapy with anticonvulsants	10	11.5–14.3	87–110	2.8–7.0
Alcohol-induced macrocytosis	7	8.8–15.6	100–125	3.0–5.7
Primary acquired sideroblastic anaemia	1	10.1	110	4.1
Subacute erythraemic myelosis	2	10.3, 13.2	104, 110	6.6, 5.6
Acute myeloid leukaemia	3	8.9–11.9	95–106	4.1, 4.7, 3.6
Chronic myeloproliferative disorder with thrombocythaemia	1	8.8	101	4.5
Myelomatosis	2	10.5, 14.4	105, 99	5.8, 3.9
Lesch-Nyhan syndrome (hemizygote)	1	11.0	119	2.0
Ineffective erythropoiesis preceding pure red cell aplasia	1	5.9	107	[illegible]

Macrocytosis due to chronic alcoholism. Marrow aspirates from 45 patients with a macrocytosis associated with chronic alcoholism (Hb 8.8–18.9 g/dl, MCV 100–116 fl) were investigated. All the patients drank at least a third of a bottle of spirits or its equivalent in wine or beer per day. Eleven of these patients were anaemic (Hb levels <13.1 g/dl in men and <12.0 g/dl in women). Erythropoiesis was mildly megaloblastic in 14 of the 45 patients, and was considered to be essentially normoblastic in the remainder. Ten of the normoblastic marrows showed greater dyserythropoietic changes than the other 21 and one of these ten samples also showed sideroblastic erythropoiesis.

Eight of the 45 chronic alcoholics gave dU-suppressed values above 8.6% and all of these 8 cases showed megaloblastic erythropoiesis. Mild megaloblastic changes were also found in 6 of the remaining 37 patients with dU-suppressed values within the 95% confidence limits for haemato-

Table IV Cases with subnormal serum vitamin B_{12} levels in which the marrow cells gave normal dU-suppressed values

Case	Hb g/dl	MCV fl	Serum B_{12} ng/l	Erythropoiesis	dU-suppressed value,	Diagnosis
LW[1]	13.5	89	93	N	3 6	clinically normal
EC[1]	15.2	84	140	N	6.1	hypertension
LH[1]	12.7	101	125	M	5.9	epileptic, on anticonvulsants[2]
RN	9 7	102	105	N	3 1	alcoholic infected amputation stump on 3 MU penicillin daily
HV[1]	14 8	86	115	N	4 1	vegan
MC[3]	9 9	65	80	N	4.5	vegan iron deficiency complicating menorrhagia

N = Normoblastic M = megaloblastic.

[1] Gave normal results with the Schilling test.

[2] Diphenylhydantoin.

[3] The serum vitamin B_{12} level remained low after the anaemia was corrected with oral iron therapy

logically normal individuals the dU-suppressed values in these 6 patients varied between 2.9 and 5 7%.

A statistically significant inverse correlation was found between the logarithm of the red cell folate level and the logarithm of the dU-suppressed value in 32 of the chronic alcoholics studied ($r = -0.67$ $p < 0.001$). An inverse correlation between these two parameters was also observed even after excluding all 9 of the alcoholics whose dU-suppressed values were above 8 0% ($r = -0.50$ $p < 0.05$). A statistically significant correlation was not found between the logarithm of the dU-suppressed value and either the MCV ($r = 0.26$ $p > 0.05$) or the haemoglobin level ($r = 0.03$ $p > 0.10$) Neither was there a statistically significant correlation between the logarithm of the red cell folate level and the MCV ($r = -0.27$ $p > 0.10$)

Table V Cases with normal serum vitamin B_{12} and subnormal red cell folate levels in which the marrow cells gave normal dU-suppressed values

Diagnosis	Cases	Hb g/dl	MCV fl	Red cell folate µg/l	dU-suppressed values, %
Alcohol-induced macrocytosis	2	12.9 14.1	105, 107	103, 70	3.6, 5.7[1]
Iron deficiency	2	10.1 11.6	68 84	106, 77	6.0, 6.9
Epileptics, on anticonvulsants	3	12.5–13.6	85–107	63 81 100	5.7[1] 7.0[1] 2.8
Renal graft, on azathioprine and cyclophosphamide	1	15.4	118	93	2.1[1]
Haemoglobin H disease	1	7.5	58	100	1.8
Congenital neutropenia	1	14.2	97	75	5.2

[1] Erythropoiesis was megaloblastic.

Combinations of iron, vitamin B_{12} and folate deficiency Four patients with a combination of vitamin B_{12} and iron deficiency 5 patients with a combination of folate and iron deficiency and 4 patients with a combination of vitamin B_{12} and folate deficiency all showed dU-suppressed values greater than 9.0%.

Patients receiving dihydrofolate reductase inhibitors. Several patients with normal MCVs and normal serum vitamin B_{12} and red cell folate levels who were receiving Septrin did not give abnormal dU-suppressed values. However 2 patients with a high MCV and megaloblastic erythropoiesis who gave abnormally high dU-suppressed values had received Septrin on the day of the marrow aspiration and during the preceding 7 days. One of these patients, with a dU-suppressed value of 26.2%, appeared to take a folate-deficient diet but had normal red cell folate and serum vitamin B_{12} levels of 116 µg/l and 310 ng/l, respectively The other patient showed a reduction in the dU-suppressed value from 31.7 to 10.2%, after stopping Septrin therapy for 2 days and was subsequently shown to suffer from pernicious anaemia.

A very high dU-suppressed value of 61.7% was given by a marrow aspirate taken 2 days after a patient with carcinoma of the maxilla was

treated with a single intravenous infusion of 1 g of methotrexate (followed 24 h later by a single intravenous infusion containing 100 mg folinic acid) This high dU-suppressed value was unaffected by the addition of 50 μg PGA per ml of marrow culture but was reduced to 34 0% by the addition of 50 μg folinic acid per ml of culture.

Patients receiving antipurines Twenty patients with megaloblastic erythropoiesis caused by antipurines were found to have normal dU-suppressed values. This group of patients included 2 cases on 6-mercaptopurine, 1 on thioguanine and 17 on azathioprine. A statistically significant correlation was not found between the logarithm of the red cell folate level and the logarithm of the dU-suppressed value in the 17 patients receiving azathioprine ($r = 0.22$ $p > 0.1$)

Epileptics receiving anticonvulsants Four out of 19 epileptics studied gave high dU-suppressed values. All 4 of these admitted to taking a poor diet and showed megaloblastic erythropoiesis prior to improvement in their diet or folate therapy The data relating to these 4 cases are summarised in table I.

Relationship between the Type of Erythropoiesis and the dU-Suppressed Value

All but 8 of the patients with abnormally high dU-suppressed values showed megaloblastic erythropoiesis. The essential details of the 8 patients in whom an abnormal dU-suppressed value was associated with normoblastic erythropoiesis are shown in table II

Table III summarises the data on patients in whom normal dU-suppressed values were associated with megaloblastic erythropoiesis.

Cases in which Subnormal Serum Vitamin B_{12} or Red Cell Folate Levels were Associated with Normal dU-Suppressed Values

Most patients with subnormal serum vitamin B_{12} or red cell folate levels gave abnormally high dU-suppressed values. The main features of the few patients in whom subnormal serum vitamin B_{12} or red cell folate levels were associated with normal dU-suppressed values are given in tables IV and V

Discussion

Abnormally high dU-suppressed values have been previously reported in vitamin B_{12} or folate-deficient patients [2, 5 9 13] and in patients re-

ceiving the dihydrofolate reductase inhibitors methotrexate [7] pyrimethamine [7] triamterene [1] and trimethoprim [2, 3] In the present study most of the marrow aspirates which gave high dU-suppressed values came from patients who were deficient in either vitamin B_{12} or folate. The cases in which high dU-suppressed values were unassociated with a deficiency of one or other of these vitamins included 1 patient who had received a single infusion of methotrexate 2 days previously and 2 patients with apparently uncomplicated iron deficiency anaemia. The explanation for the occurrence of abnormal dU-suppressed values in occasional patients with iron deficiency anaemia remains to be determined. Apart from the 2 patients with iron deficiency anaemia referred to above, 2 patients with vitamin B_{12} deficiency 1 patient with folate deficiency and 3 patients with a combination of iron and vitamin B_{12} or folate deficiency all of whom showed normoblastic erythropoiesis (table II), the other patients who gave abnormally high dU-suppressed values showed megaloblastic erythropoiesis.

Several patients with a macrocytosis induced by antipurines, cyclophosphamide or a combination of azathioprine and cyclophosphamide, 1 patient with primary acquired sideroblastic anaemia, 1 patient with a chronic myeloproliferative disorder associated with thrombocythaemia, 1 patient with ineffective erythropoiesis preceding the development of a pure red cell aplasia, 2 patients with subacute erythraemic myelosis, 1 patient with the Lesch-Nyhan syndrome (hemizygote), a proportion of the patients with alcohol-induced macrocytosis, acute myeloid leukaemia or myelomatosis and a proportion of epileptics receiving anticonvulsant therapy gave normal dU-suppressed values despite the fact that they showed megaloblastic erythropoiesis. Evidently the megaloblastic changes encountered in these patients are not caused by vitamin B_{12} or folate deficiency or by an impairment of the methylation of deoxyuridylate to thymidylate due to any other reason.

The dU-suppressed value was found to be more reliable than either the serum vitamin B_{12} or red cell folate levels in detecting vitamin B_{12} or folate deficiency Thus, whereas all patients who were considered to be vitamin B_{12} or folate-deficient (on the basis of a dietary history the MCV the nature of erythropoiesis, and the results of Schilling tests and other tests of malabsorption) gave abnormally high dU-suppressed values even when the deficiency state was complicated by iron deficiency or the anaemia of chronic disorders, several of these patients (including some with uncomplicated vitamin B_{12} or folate deficiency) had serum vitamin

B_{12} and red cell folate levels within the normal range. Four patients in whom abnormally high dU suppressed values were associated with a chronic haemolytic anaemia (reticulocyte counts 10–16%) also had red cell folate levels within or above the range observed in normal individuals and in these patients this discrepancy probably resulted from the high folate content of reticulocytes and young red cells.

Some patients with subnormal serum vitamin B_{12} and red cell folate levels gave normal dU-suppressed values (table IV V) indicating that the marrow cells of these patients did not suffer from the metabolic consequences of vitamin B_{12} or folate deficiency This group of patients included 1 patient on high doses of penicillin in whom the low serum vitamin B_{12} level was due to a penicillin induced inhibition of the growth of the assay organism *L. leichmanii* and 2 patients who were vegans. The data in the 2 vegans indicate that at least some vegans manage to supply their marrow cells with adequate quantities of vitamin B_{12} despite their vitamin B_{12}-poor diet and subnormal serum vitamin B_{12} levels.

Only 18% of the chronic alcoholics with macrocytosis appeared to be folate-deficient on the basis of their giving both a high dU-suppressed value and a low red cell folate level the remaining 82% gave normal dU-suppressed values and usually had normal red cell folate levels. These results suggest that the macrocytosis shown by chronic alcoholics is at least partly and in most cases entirely caused by a folate independent effect on erythropoiesis. An alternative and less likely possibility which is also consistent with the present data is that in most alcoholics the macrocytosis is entirely caused by an intermittent alcohol induced effect either on the supply of folate to bone marrow cells or on the metabolism of folate within these cells and that this effect only operates when the blood alcohol level is high On this hypothesis all those alcoholics who gave normal dU-suppressed values would be expected to have given abnormal values had their marrow cells been studied at a time when the blood alcohol levels were at their highest. The explanation for our observations that chronic alcoholics with normal dU-suppressed values showed a significant but weak inverse correlation ($r = 0.50$ $p < 0.05$) between the logarithm of the red cell folate level and the logarithm of the dU-suppressed value and that 2 other groups of patients with normal dU-suppressed values (one with azathioprine-induced megaloblastosis and the other with the anaemia of chronic disorders) did not, is uncertain One possible explanation for these findings is that they result from a dampening of the diurnal and long-term fluctuations in the serum folate level in chronic alcoholics.

Previous authors have reported that the addition of 1 μg cyanocobalamin/ml of marrow culture together with the deoxyuridine, causes a significant reduction in the high dU-suppressed value given by vitamin B_{12}-deficient marrow cells but does not affect the high dU-suppressed values given by folate-deficient marrow cells, and have claimed that this difference can be successfully exploited to distinguish between vitamin B_{12} and folate-deficient patients [2, 5 6, 11] However in the present study the addition of 1 μg cyanocobalamin/ml of marrow culture not only failed to cause a significant reduction (i.e. of 15%/ or more) in the dU-suppressed value in 14 / of vitamin B_{12}-deficient patients but also caused a significant reduction in the dU-suppressed value in an occasional patient considered to be primarily folate-deficient.

A statistically significant inverse correlation was found between the red cell folate level and the percentage reduction in the dU-suppressed value induced by the addition of 10 μg PGA/ml of marrow culture together with the deoxyuridine in a group of patients with uncomplicated folate deficiency ($r = -0.56$ $p < 0.01$) Furthermore, this concentration of PGA caused a reduction of 15 / or more in the dU-suppressed values of most but not all folate-deficient marrows. However as a similar effect was seen in 57%/ of vitamin B_{12}-deficient marrows these two deficiency states cannot be distinguished on the basis of a differential effect following the addition of 10 μg PGA/ml of culture

Four epileptics on anticonvulsants showed high dU-suppressed values. In an earlier paper we concluded that the high dU-suppressed value shown by the epileptic CM(a) (table I) might be caused by a drug-induced interference with the methylation of deoxyuridylate rather than by folate deficiency because the addition of 10 μg PGA/ml of marrow culture together with the deoxyuridine did not lead to any reduction in the dU-suppressed value [12] This conclusion cannot be supported any longer as the present data reveal that a few folate-deficient patients do not show any correction in their high dU-suppressed values after the addition of this concentration of PGA. In fact, case CM was seen again recently (i.e. 1 year later) with a severe megaloblastic anaemia and on this occasion gave a high dU-suppressed value which was reduced by more than 50%/ in the presence of 10 μg PGA/ml of marrow culture (table I, case CM(b)). She admitted to taking a folate-deficient diet and, following therapy with 5 mg folic acid daily for 3 weeks, gave a normal dU-suppressed value despite the continuation of her anticonvulsant therapy (table I, case CM(c)). These findings in case CM strongly suggest that all 3 of the other epilep-

ties who gave high dU-suppressed values (table I) also suffered from a folate deficiency which was at least partly of dietary origin.

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Transfer of Bovine J Blood-Group Activity to Human Erythrocytes *in vitro*

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Key Words. Blood group · Bovine J blood group · Erythrocyte transformation · Transfer of blood group determinant

Abstract. The bovine J determinant is transferred from bovine serum nonlipid fraction to human erythrocyte membrane lipid by an incubation procedure. The transferred J determinant is detected in the total lipids extracted from transformed human erythrocytes by an inhibition test in the bovine J system. It is also detected by cross-reacting human anti-A sera after treatment of human cells with papain prior or subsequent to transformation.

It has been shown recently [1–3] that the bovine J blood-group determinant is transferred from a serum protein onto the bovine erythrocyte membrane by incubation *in vitro.* Even though the carrier of J activity is a lipid-free serum protein (probably a glycoprotein) [4], the transferred J activity is found only in the lipid fraction of erythrocyte membranes. Thus, the J determinant (probably a carbohydrate moiety) must have been split from the J serum glycoprotein and transferred to a lipidic receptor (which may be a glycosphingolipid) at the erythrocyte membrane. It was suggested that an enzyme system located at the bovine erythrocyte membrane is responsible for the transfer of the J determinant.

Furthermore, it was shown by other workers that human blood-group activities can be transferred to human erythrocytes by incubation with blood-group active sera [5, 6] or lipids [7, 8] *in vitro.* However, it was not reported whether human blood-group determinants are transferred from serum glycoproteins to erythrocyte membrane lipids.

This paper will show that the J bovine blood-group determinant is

transferred from bovine lipid free protein onto human erythrocyte membrane lipid.

Materials and Methods

Bovine blood was obtained from the local slaughter house, human blood from the Göttingen blood bank. Preparation of human erythrocytes and bovine serum was done in the usual manner

Total lipids were extracted from original and transformed human O cells with chloroform-methanol and purified as described previously [9]. Total lipids were similarly extracted from bovine J serum, and the precipitate obtained after this extraction procedure was carefully washed with chloroform-methanol It served as the lipid-free donor of the J determinant.

Washed erythrocytes were treated with neuraminidase (EC 3.2.1.18) [10] or with papain (EC 3.4 4.10) [11] prior or subsequent to transformation.

Incubation of human O and B cells with the lipid-free donor was performed as previously described [1] briefly a suspension of lipid-free donor in isotonic saline was incubated with a suspension of human erythrocytes under constant slight shaking at 37 °C for 24 h. Then the erythrocytes were washed and treated further as indicated. All experiments were repeated several times.

Cellular J activity was tested by immunohemolysis in the homologous (bovine) J system, cellular A and B activity by hemagglutination in the human AB system. J activity of total lipids was assayed by immunological hemolysis inhibition tests as described in a previous paper [12]. Agglutinations by agglutinin of *Helix pomatia* (anti-A_{HP}) and by lectin of *Dolichos biflorus* (anti-A_{Db}) were performed in slide tests using a 2% suspension of erythrocytes.

Results

Human O erythrocytes were incubated with J active nonlipids prepared from bovine J containing serum. In order to test whether or not the incubation has succeeded in transferring the J determinant onto the human erythrocyte membrane, bovine anti J serum is not directly applicable because of its species specificity However the total lipids extracted from the human erythrocytes subsequent to incubation can be assayed for J activity by application of an immunohemolysis inhibition test in the bovine J system. As shown in figure 1 the total lipids of human erythrocytes, thus treated, were J positive they showed no reaction in the J system when there was no previous incubation with J containing nonlipids, as reported previously [13]

Since there is a cross-reacting relationship between bovine J and human A blood groups [13] it should be possible to demonstrate the result

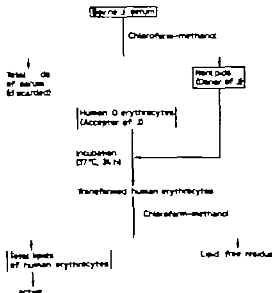


Fig 1 Schedule showing the procedure of incubation of human O erythrocytes with lipid-free residue of bovine J serum.

ing transfer of J determinant by reaction with human anti-A serum. He magglutination tests of human O or B erythrocytes thus transformed resulted in very weak reactions in more than 20 transfer experiments. When however the transformed human erythrocytes are treated with neuraminidase or papain the cells become highly active towards human anti-A serum while no activity is noticed towards anti-B serum (table I). The same is true when the erythrocytes are pretreated with neuraminidase or papain and the transfer of J is subsequently performed.

By treatment with neuraminidase, the T receptor and the HP receptor at the human erythrocyte membrane are made available for anti-T and anti-A_{HP}, respectively [14]. Since every human serum contains anti-T such cells are agglutinated by any human serum. Interestingly the T receptor is no longer reactive when human erythrocytes are incubated with J containing nonlipid of bovine serum before or after treatment with neuraminidase. However human erythrocytes thus treated are agglutinated by agglutinin of *Helix pomatia* (anti A_{HP}). Lectin of *Dolichos biflorus* (anti-A_{hel}) does not react with any of the cell types listed in table I

transferred from bovine lipid-free protein onto human erythrocyte membrane lipid

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Since there is a cross-reacting relationship between bovine J and human A blood groups [13] it should be possible to demonstrate the result

The transferred J determinant seems to be hidden partially by polypeptide chains. Thus, cross-reacting human anti-A sera give weak agglutinations only; they are, however, strongly agglutinating if the cell surface polypeptides are hydrolyzed by the action of papain before or after the transfer of J, thus making the transferred J determinant available for the anti-A antibody.

After treatment of human O erythrocytes with neuraminidase, the T receptor becomes available for anti-T which is contained in all human sera including anti-A and anti-B sera. If the human cells have been transformed by incubation with bovine J prior or subsequent to neuraminidase treatment, the T determinant is no longer detectable by anti-T; therefore, no reactions with anti-B sera are noticed (fig. 1), while reactivity with anti-A sera is obviously due to the known cross-reactivity of anti-A with bovine J. In contrast, the HP receptor becoming available for anti-A_{HP} after treatment with neuraminidase remains detectable with anti-A_{HP} in transformed human cells.

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Table I Agglutination test of original and treated human O red cells with human antisera (anti-A and anti-B) and with natural agglutinin of *Helix pomatia* (anti-Ahp)

	Anti A	Anti-B	Anti-Ahp
Original cells	0	0	0
Cells incubated with J	1 2	0	0
Cells + papain	0	0	0
Cells incubated with J + papain	6	0	0
Cells + papain incubated with J	6	0	0
Cells + neuraminidase	7.5	6	8
Cells incubated with J + neuraminidase	5	0	8
Cells + neuraminidase incubated with J	5	0	8

The results are reported in score numbers [16], 8 meaning complete agglutination, 0 no agglutination at all other numbers indicate intermediate reactions. All values are means of duplicates.

Discussion

The above results indicate that the bovine J determinant is transferred from a nonlipid fraction of J containing bovine serum to a lipid of human erythrocyte by incubation *in vitro* Since all the lipids of mature mammalian erythrocytes reside in their membranes [15] the transferred J activity is located in the red cell membrane. When studying the transfer of J from a bovine serum nonlipid to bovine erythrocytes it was suggested that an enzyme located at the erythrocyte membrane may be involved in the transfer process [1–3] This also seems to be true in human erythrocyte membrane

The transferred J determinant seems to be hidden partially by polypeptide chains. Thus, cross-reacting human anti-A sera give weak agglutinations only; they are, however, strongly agglutinating if the cell surface polypeptides are hydrolyzed by the action of papain before or after the transfer of J, thus making the transferred J determinant available for the anti-A antibody.

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Refractory Sideroblastic Anemia Secondary to Autoimmune Hemolytic Anemia

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Key Words. Autoimmune hemolytic anemia · Immunosuppressor · Sideroblastic anemia

Abstract. A 75-year-old woman was hospitalized with autoimmune hemolytic anemia. During a period of 22 months the patient had six hemolytic crises which responded to treatment with prednisone and azathioprine. During the last admission the patient presented a sideroblastic anemia with 98% of 'ring sideroblasts' in the bone marrow. This association has never, to our knowledge, been reported before. It is possible that the immunosuppression played a definite role in the development of this sideroblastic anemia.

An aplastic crisis can complicate the evolution of an hemolytic anemia of various origins including autoimmune hemolytic anemia (AIHA) [2]. However, the appearance at the same time of ring sideroblasts in the bone marrow has not been reported, to our knowledge, during an AIHA. The case recorded here describes the evolution of an AIHA, on immunosuppressor therapy, into a refractory sideroblastic anemia (RSA).

Case Report

A 75-year-old woman was admitted to the hospital in 1971. She had been suffering from asthenia for 2 weeks. During the clinical examination, the patient was pale with jaundiced sclerae without either palpable adenopathies or palpable spleen.

The following data led to the diagnosis of AIHA: hemoglobin (Hb), 8.1 g/dl; reticulocytes, 10.5%; platelets, 185,000/mm³ and white cell count (WBC), 4,600/mm³ with a normal distribution. The bone marrow showed a high cellularity with erye

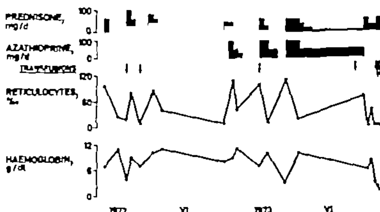


Fig. 1. Hematological data during course of illness in relation to treatment.

loid/erythroid hyperplasy. Total bilirubin, 31.3 mg/l; lactic dehydrogenase, 300 UI/l; haptoglobin, 0 mg/l; serum iron, 230 µg/dl; folic acid, 6 ng/ml [normal (N): 7–16 ng/ml] and vitamin B_{12}, 95 pg/ml (N: 200–1,000 pg/ml). The direct Coombs test was positive and showed an IgG specificity. Antinuclear factor, anti-DNA, antithyroid and antigastric antibodies were negative. Chromium red cells survival was 17 days (N, 25–32 days). The evolution of the disease and its treatment are summarized in figure 1. During each hospitalization the patient regulary received vitamin B and folic acid. During the last month of the evolution, the hematologic pattern changed dramatically: Hb, 6.7 g/dl; hematocrit (Ht), 20%; reticulocytes, 0.5 ‰; WBC, 2,550/mm; platelets, 48,000/mm³; serum iron, 210 µg/dl. The peripheral blood smear showed a red cell hypochromia. A treatment of 60 mg prednisone/day and 2 transfusions did not significantly modify the hematologic data (Ht, 23 %). A toxic effect of azathioprine was suspected but a bone marrow aspiration was refused by the patient. Eight days after discontinuing this medication, the reticulocytes rose to 71 (Ht, 32%).

Two weeks later the patient was hospitalized again with the following hemogram: Hb, 4 g/dl; reticulocytes, 0.2‰; WBC, 8,700/mm³; myelocytes, 87/mm; metamyelocytes, 348/mm³. Total bilirubin, 39.2 mg/l; lactic dehydrogenase, 370 UI/l; serum iron, 200 µg/dl; vitamin B_{12}, 2,500 pg/ml; folic acid, 50 ng/ml. The bone marrow showed a high cellularity with a M/E ratio 1/1. The erythroid cells were hyperplastic and megaloblastic at all stages of maturation and showed an important dyserythropoiesis. The iron coloration showed abundant stores and 98% ring sideroblasts. In spite of transfusions and corticoids (prednisone 60 mg/day) the patient died 4 days later. The autopsy confirmed the bone marrow erythroid hyperplasia extending to the femoral head and the skull, the ring sideroblasts (about 100%), the iron-rich stores and the absence of myelofibrosis. It showed, moreover, a splenomegaly (330 g), a splenic and hepatic hemosiderosis, a vesicular lithiasis and an atrophy of the gastric mucosa.

Discussion

The diagnosis of RSA during the terminal evolution was based on resistance to treatment, a low reticulocyte count, a high serum iron, a megaloblastic bone marrow with nearly 100% ring sideroblasts and an erythroid hypercellularity affecting the femoral and the skull as well.

A phase of so-called 'aplastic' anemia with reticulocytopenia is not exceptional in the evolution of an AIHA [2] Such an evolution occurs in other well known types of hemolytic anemias hereditary spherocytosis [4] falciform anemia [9] new born child's hemolytic disease [6] etc.

However the presence of ring sideroblasts is an unusual observation described only in 2 cases of hereditary spherocytosis and in one of elliptocytosis [8] The etiology of these 'aplastic' crises is not known. An infection or a vitamin B_{12} or folic acid deficiency have been incriminated. In the presented case, neither of these causes were present, at the time the RSA was diagnosed.

However a few data might have suggested an association with Biermer's anemia. low B_{12} serum value at the beginning of the illness, gastric atrophy and achlorhydria (even with pentagastrin) Schilling test, 11.3% (N 20±8%), corrected to 19 6% by the intrinsic factor The association of Biermer s anemia and an AIHA is well known [3] and the appearance of ring sideroblasts in B_{12} of folic acid deficiencies is well established [5] However they disappear with a substitutive therapy and the correction of the megaloblastic anemia. Here, the RSA was certainly not a manifestation of a Biermer's anemia because it appeared after 1 year repeated perenteral administration of vitamin B and folic acid which were both found very high in the patient s serum (vitamin B_{12}, 2,500 pg/ml folic acid, 50 ng/ml) when the RSA was recognized. Our patient had been treated for more than 1 year with azathioprine. It is possible that this immunosuppressor played a role in the hematologic anomalies seen KHALEELI *et al.* [7] reported, indeed, the appearance of a sideroblastic anemia, then of a leukemia in 4 myelomatous patients on busulphan therapy Moreover in the group of sideroblastic anemias associated with other diseases such as rheumatoid arthritis [8] systemic lupus erythematosus [1], periarteritis nodosa, Hodgkin s disease, myeloproliferative syndromes [5] and lymphoma [11] many patients have been treated with immunosuppressors. TCHERNIA *et al.* [10] described 11 patients with acute myeloblastic leukemia after immunosuppression therapy for primary nonmalignant disease

In the etiology of the RSA these drugs could aggravate an enzymatic deficiency or induce a mutation or clonal degeneration of the stem cell terminating in a RSA or a leukemic evolution

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A New Approach to the Diagnosis of β^+ Thalassaemia

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Key Words. Globin chain separation · Isoelectric focusing · β^+ Thalassaemia · Traces of HbA

Abstract. By use of isoelectric focusing in polyacrylamide gel rods we were able to detect traces of HbA (approx. 1 %) as sharp and discrete band. By overloading the gel considerable amounts of HbA (slightly contaminated with HbF) could be detected and isolated. The focused HbA was retrieved from the gels, separated from the carrier-ampholytes and concentrated by one-step electrophoresis technique. With ^{3}H-leucine-labelled haemolysates, after globin chain separation on CM-cellulose, an increase of the β-chain counts relative to γ-chain counts was obtained. The study of two cases of high HbF homozygous β-thalassaemia has demonstrated that this technique may be valuable tool in detecting minute amounts of HbA mainly in high HbF β-thalassaemias.

Introduction

The β-thalassaemias are a heterogeneous group of inherited disorders characterized either by reduced β-chain synthesis (β^+-thalassaemia) or total absence of β-chain production (β^0-thalassaemia) [8, 34, 35, 37]. The precise molecular defect in the β-thalassaemia has not yet been clarified [37]. However, in the β^+ type it is certain that there is a deficiency of the β-chain mRNA [3, 17, 18, 23, 24]. Regarding the β^0-thalassaemia, although total absence of β-mRNA has been found [27], Clegg and Weatherall [8] and Weatherall and Clegg [35] suggested that in some cases, at least, a highly unstable mRNA may possibly be synthesized. Indeed, recently Pritchard *et al.* [26] had indications that small amounts of β-chain mRNA are present in the reticulocytes of the Ferrara β-thalassaemia homozygotes.

The introduction of new techniques able to detect traces of HbA is expected to contribute considerably to the diagnosis and further research on β^0 thalassaemia [35] Although isoelectric focusing (IEF) in polyacrylamide gel rods [1 4 11 13 14 20 28 29 31 38] is a method of high resolution and sensitivity [13 15 30] it has not yet to our knowledge, been used for studies on β thalassaemia.

In this work, attempts are presented to develop a technique for the detection of minute amounts of HbA or newly synthesized β-chain, based on both IEF [13] and globin chain separation on CM-cellulose [7]

Methods

Haematological Methods and Hb Analysis

Haemoglobin synthesis was studied in peripheral blood of two high HbF β thalassaemic homozygotes in centrifuge tubes containing 500 IU of heparin Samples of cord blood were obtained from the placenta immediately after delivery Haematological studies were made using standard techniques [10] Electrophoresis of Hb was performed in starch gel using a Tris-EDTA borate buffer pH 8.6 [34] HbA_2 was determined by cellulose acetate electrophoresis [12] and HbF was quantitated by alkali denaturation [32]

Haemoglobin Synthesis

Haemoglobin synthesis was studied in peripheral blood of two high HbF β-thalassaemi homozygotes. The plasma was removed by centrifugation and the cells were then washed twice in reticulocyte saline (RS, 0.13 M NaCl, 0 005 M KCl, 0.007 M $MgCl_2$-6 H O). The cells were resuspended in RS and one more centrifugation was performed at 3 000 g for 20 min at 4 C in order to increase the concentration of reticulocytes in the top layer of the packed cells 0.5 ml of cells was removed from the top layer of packed cells rich in reticulocytes. These cells were incubated for h with 300 Ci ^{3}H-leucine in the medium of LINGREL and BORSOOK [21] modified for use with human cells as described by WEATHERALL et al [36] The incubation was stopped by adding a large volume of ice-cold RS. The cells were then washed four times in the same solution at 4 °C. Lysates were prepared by adding 4 vol 0.01 M KCN After vigorous shaking the sample was allowed to stand for 10 min at room temperature Then, the membranes were removed by centrifugation at 38,000 g for 45 min. Furthermore, the preparation of globin, separation of globin chains and determination of radioactivity were performed as described by CLEGG et al [7], WEATHERALL et al. [36] CHALEVELAKIS et al [5 6]

IEF of Haemoglobin in Polyacrylamide Gel Rods and Elution of HbA by Electrophoresis

IEF of Hb was performed as described by DRYSDALE et al [13] with slight modifications. The method is briefly as follows. the gel contained 5% acrylamide (stock

solution 40% containing 2% 2N-methylene bisacrylamide), 1% ampholyte, pH 6–8 (40% solution w/v LKB Producter Bromma, Sweden), 4% glycerol and 0.17% 4N-tetramethylethylenediamine. After the solution had been degassed under tap vacuum, 0.06% ammonium persulphat was added and the solution was quickly poured with Pasteur pipette in polyacrylat tubes (10×0.3 cm inner diameter). The gels were allowed to polymerize at room temperature for 2–3 h then 20–100 µl of loading solution was layered on the top of each gel.

In our experiments the loading solution consisted of 1 vol haemolysate to 3 vol M/20 phosphat buffer pH 6.63, but this ratio may vary widely. The phosphate buffer contained 0.25 ml ampholytes and 3 ml glycerol in final volume of 10 ml. After 4 h running HbA_{II} focused as sharp and discret band between HbF_I and F_{II}, closer to F_I [13] (fig. 1).

In artificial mixtures prepared by mixing equivalent solutions of HbA (isolated by DEAE Sephadex) [11] and HbF (haemolysates from β^0-thalassaemia, case II) containing progressively decreasing amount of HbA, the latter was clearly visible even in solutions containing 1% HbA. For the isolation of HbA, haemolysates labelled with ^3H-leucine (see above) were used and the gels were overloaded with 100 µl of loading solution. Finally the gels were removed from the tubes and the segments carrying the focused HbA were pooled and then homogenized. The homogenate was packed in one gel unit of multiphase electrofocusing column [33] and electrophoresed at current of 10 mA and final voltage of 38 V against dialysis membrane, permeable only to ampholytes. Complete retrieval of HbA in concentrated form was obtained after h. I scal-up experiments, using carrier-ampholytes of narrower pH range in multiphase column, sufficient amounts of HbA can be separated and subsequently retrieved by electrophoresis [33]. Such experiments are presently in progress. T the isolated HbA contained in 0.5 ml solution, an equal volume of cold haemolysates consisting of 50% HbF and 50% HbA was added in order to provide sufficient unlabelled carrier for further separation of globin chains.

Results

The Hb pattern obtained by IEF from normal and cord blood haemolysates (fig. 1) was as described by DRYSDALE *et al.* [13]. In high HbF β^+-thalassaemia the pattern is similar to that obtained from cord blood. In the β thalassaemia the band of HbA is missing. The HbF is resol ed in two components, the main F_{II} and the minor F_I which is more acid. HbA is also resolved in the main A fraction and the modified minor A_I [16]. HbA_{II} focused between F_{II} and F_I and ery close to F_I with a difference of 0.05 pH units [13] (fig. 1). In artificial HbF and HbA mixtures the resolution between HbA and HbF was influenced both by the amount of the applied Hb and the F_I/A ratio. It was found that the



Fig 1 Isoelectric fractionation of haemoglobins derived from (1) normal adult subject (2) case (I) of high HbF β-thalassaemia Fraction X corresponds to the abnormal component X in DEAE-Sephadex and CM-cellulose chromatography (cf fig 4).

Fig 2 Isoelectric fractionation of haemoglobins derived from (1) normal adult subject (2) case (II) of high HbF β thalassaemia (3–5), case (II) containing progressively increasing amounts (6, 7 15%) of added HbA_{II}

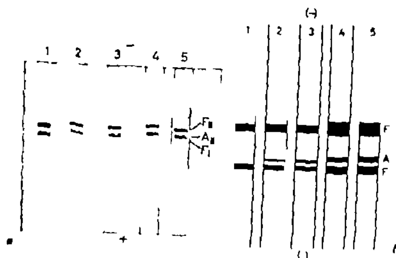


Fig 3 *a* IEF of Hb fractions from (1) haemolysate of case II (2, 3) haemolysate of case II containing 1 and 2% respectively of added HbA (4 5), a in (2, 3) but with double loading volume *b* Schematic illustration of the Hb pattern shown in figure 3a, as observed directly at the end of the run



Fig. 4 DEAE Sephadex chromatography of haemolysate derived from case I.

most successful illustration of HbA band was obtained when the applied amount of Hb in each gel was approximately 200 μg and the HbA fraction was less than 20%. Under these conditions we were able to defect as little HbA as 1 / from artificial Hb mixtures (fig. 2, 3a, b).

Study of the Cases of High HbF β-Thalassaemia

First case Male aged 20 The Hb pattern was. F = 75%, A = 4.7% and the residual 20.3% was expected to be HbA. I starch-gel electrophoresis small amounts of HbA were seen. I DEAE Sephadex chromatography the Hb pattern was: (F + F_1) = 78%, A_2 = 5% , A = 9% with the appearance of an abnormal component, 8% resembling chromatographically Hbs Lepore (fig. 4) The globin chain separation on CM-cellulose showed, in the β-chain region, hardly distinguishable β-chain peak against background of large amounts of γ-chain with ratio of respective radioactivities β-chain/γ-chain = 0.34 (fig 5a). The γ- and β-chains after dialysis against large volume 0.5% solution of formic acid and freeze-drying and in the presence of sufficient amounts of cold normal adult and cord blood globin was rechromatographed on CM-cellulose. This procedure provided more cold β-chain as carrier of its own counts and resulted in far better separation between the γ- and β-chain counts, but with no improvement of the β-chain/γ-chain radioactivity ratio (fig 5b). After isolation of the focused HbA from the acrylamide gels and separation of the globin chains on CM-cellulose (see Methods) we obtained higher values of β-chain radioactivity relative to γ-chain, namely ratio of counts β-chain/γ-chain = 1.6 (fig 5c). The presence of γ-chain is due to inevitable contamination of HbA by the closest fraction HbF_1 during removal of the HbA fraction.

Second case Female aged 18 The Hb pattern was: HbF = 95% traces of HbA_2. All the conventional methods and the more sensitive technique of IEF failed to detect any HbA or β-chain from peripheral blood haemolysates (fig. 3a, b). Thus we concluded that the patient suffered from β -thalassaemia. In order to exclude the presence in the IEF pattern of non-visible traces of HbA this technique was repeated using hot haemolysates in which some cold HbA was added as carrier. The

focused HbA was isolated and the globin chains were separated, but no newly synthesized β-chain was detected (fig. 6a–c).

Discussion

β Thalassaemia is characterized by total absence of β-chain synthesis [34 37] This type of β thalassaemia has mainly been described in Ferrara [2, 9 25] and the Mediterranean [36] In Greece recent work has indicated that the occurrence of β thalassaemia may be extremely high KATTAMIS *et al* [19] on 75 never transfused cases of β/β or $\beta/\beta\delta$ genotype found that the mean level of HbF was about 73 and 75% respectively In addition in more than 80% of these cases the HbA was completely absent

The primary molecular defect in β°-thalassaemia remains obscure and at present the likelihood of its being a heterogeneous disorder cannot be excluded [35] It has been suggested that in β° thalassaemia a reduced amount of HbA may be synthesized only in the bone marrow thus the Hb synthetic pattern might be similar to the pattern of HbsA$_{F}$, Lepore and anti Lepore (Miyada) Therefore each untransfused case of high HbF β thalassaemia must be examined for traces of HbA and newly synthesized β-chain both in peripheral blood and bone marrow

In the present work our approach to this problem has been based on IEF in polyacrylamide gels [13 28 29] This technique resolves substances which differ only by 0 005 pH units it is also capable of detecting traces of protein as small as 0 2% HbA$_{2}$ in cord blood [13] Furthermore GAINER [15] lowered the limit of detecting proteins down to the level of 10 ^{16} g by use of a microscale IEF method.

These advantages along with our finding that in artificial mixtures of HbF and HbA the latter was clearly visible as a sharp and discrete band even at the low limit of 1%, show that this method may be of value in the differentiation of β from β -thalassaemia

An additional advantage of the suggested technique is that it can be

Fig 5 Chain separation on CM-cellulose of H leucine-labelled globin derived from peripheral blood haemolysate of case I (a). The (β +) chromatographic fractions (profile a) which were rechromatographed on CM-cellulose after adding sufficient amount of globin A as carrier (b) Eluted HbA (contaminated by HbF$_{I}$) after its focusing in polyacrylamide gels which were loaded with haemolysate from case I (c).

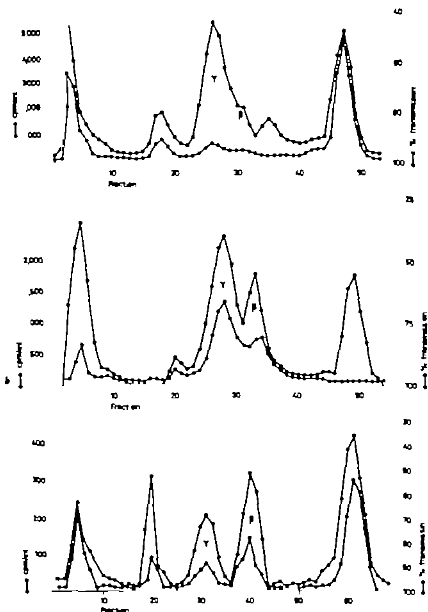


Fig. 5

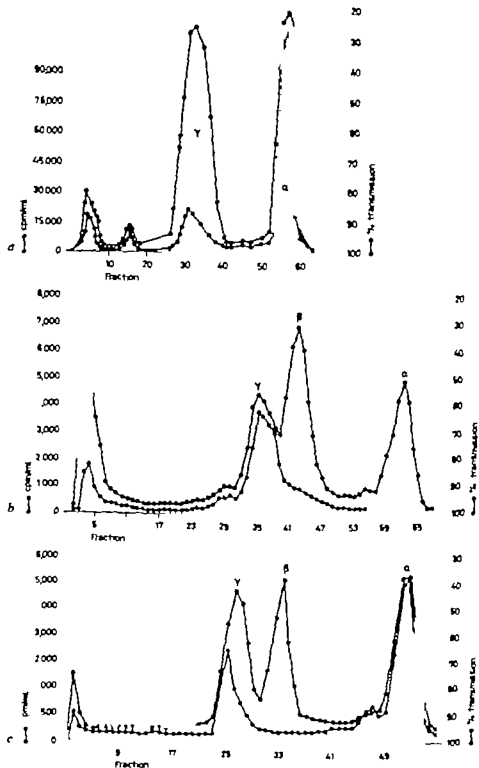

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Fig. 6

applied for the detection of pre-existing HbA as well as newly synthesized β-chains at the biosynthetic level. Thus in the first case of high HbF β-thalassaemia we isolated the focused HbA (fig. 1) and after globin separation on CM-cellulose we obtained a remarkable segregation of the relatively minute amount of β-chain radioactivity from the large background of the γ-chain counts (fig. 5a–c). A significant increase of the β-chain/γ-chain ratio occurred, namely from 0.34 in the original separation, to 1.6. In the second case of high HbF β-thalassaemia we were unable to detect HbA or β-chain counts at the biosynthetic level using peripheral blood. Consequently this case must be a β^0-thalassaemia as far as the peripheral blood is concerned.

The wide application of the described technique in every new untransfused case of high HbF β-thalassaemia is expected to reduce significantly the reported high values [19] of incidence of β^0-thalassaemia in some populations. In addition, biosynthetic studies for the detection of traces of β-chain radioactivity in β^0-thalassaemic bone marrow may substantially contribute to the elucidation of the molecular defect in this condition.

Another potential use of IEF combined with globin chain separation would be in the detection and isolation of HbA during the first months of fetal life, a significant contribution to the intrauterine diagnosis of thalassaemias.

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Fig. 6. a–c As in figure 5 but using haemolysate from case II. Note the complete absence of β-chain synthesis.

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Haemoglobin H Disease and Pregnancy in a Malaysian Woman

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Key Words. Haemoglobin H · Pregnancy · Uncommon · Haemolytic anaemia · Malaysia

Abstract. A case of haemoglobin H (HbH) disease associated with pregnancy is presented and discussed in the light of reports in the literature. The variable symptomatology is commented upon, although mild to moderate chronic haemolytic anaemia seems to be constant feature. The roles of folic acid supplements and of splenectomy, the avoidance of oxidant drugs, and the mode of inheritance in HbH disease are briefly commented upon. Available reports indicate that HbH disease probably has no adverse effect on pregnancy. However, the association of the two conditions is uncommon, and reports are too few, therefore, to allow definite conclusions on the outcome in all instances.

Haemoglobin H (HbH) disease is an α-thalassaemic disorder associated with the presence of unstable haemoglobin with high oxygen affinity which has the structure of a tetramer of four β-polypeptide chains of globin and associated haems. Its relatively high incidence in South-East Asia, the Middle East, Greece and Cyprus, and its rarity elsewhere has been commented upon [13], although cases may be expected in countries with immigrant populations from high incidence regions.

Pregnancy in HbH disease is not commonly encountered. Only eight pregnancies in 3 patients with established HbH disease have been described so far, and these have indicated that the clinical progress during pregnancy is usually favourable [4, 9, 15].

We report here a case of HbH disease associated with pregnancy in a

Malaysian Chinese woman seen at the University Hospital, Kuala Lumpur Malaysia.

Case History

N P L., a 27 year-old Chinese lady was first seen at 24 weeks pregnancy on 8-4-75 In 1972, she had uterine evacuation for incomplete abortion at 6 weeks gestation, 2 units of blood were transfused. In 1973 she had a normal delivery of a full-term live female infant weighing 2,640 g after an uneventful pregnancy She was not anaemic and required no transfusion. The infant was not anaemic or jaundiced.

In this pregnancy 7 antenatal visits were made. The uterine fundus increased steadily in height. Her weight gain of 0.37 kg per week was satisfactory [11]. She had no ankle oedema and her blood pressure was normal (110/60 to 130/80 mm Hg). She had no jaundice or hepatosplenomegaly

Standard methods were used for haematological investigations [3] and revealed a haemoglobin of 8.6 g/100 ml haematocrit (PCV) of 29%, and mean corpuscular haemoglobin concentration (MCHC) of 29.6%/ The reticulocyte count was 3.2%. Platelet and white cell counts were normal The peripheral blood film showed anisocytosis, poikilocytosis, marked anisochromia and hypochromia, microcytosis, elliptocytosis, presence of target cells and some polychromasia. HbH inclusions were found in 62% of the red cells in the reticulocyte preparation after incubation with new methylene blue. Serum iron was 286 (UIBC 17 TIBC 303) µg/100 ml, serum folate 38.5 ng/ml and vitamin B_{12} 516 pg/ml.

Haemoglobin electrophoresis of the red cell haemolysate on cellulose acetate at pH 8.6 gave a main zone of HbA (80%), reduced HbA (1.5%) and raised non-haem protein (4%), together with fast fractions of HbH (8%) and Hb Bart's (6.5%). The alkali-resistant haemoglobin was 2% of the haemolysate and acid elution of ethanol fixed films at pH 1.5 showed varying amounts of resistant haemoglobin in 3.9%/ of red cells. Red cell G6PD activity was normal.

Other investigations, blood group B, rhesus-positive blood VDRL negative; no excess of urobilinogen and no bilirubin in the urine, and stools were negative for ova and cysts. Liver function tests were normal

Her pregnancy progressed uneventfully Repeat haemoglobin levels at 33 and 37 weeks were 8.6 and 9.0 g/100 ml respectively The other haematological indices showed no changes. She was put on prophylactic folic acid (5 mg t.i.d.) during her pregnancy

Examination of her husband's blood revealed normal haematological indices. Haemoglobin H inclusion bodies were absent and haemoglobin electrophoresis revealed normal adult haemoglobin A there was no haemoglobin H. Liver function tests, serum iron, folate and vitamin B_{12} levels, and red cell G6PD activity were all within normal limits.

On 24-7 75 at term, labour was induced by amniotomy and oxytoxin drip The liquor amnii was clear She had normal delivery of a live male infant weighing 3,050 g after a labour of 4 h 51 min. Intravenous ergometrine 0.5 mg was given at

delivery of the anterior shoulder. Blood loss at delivery was about 100 ml. The placenta weighed 380 g and showed no abnormality. Immediate postpartum maternal observations were normal.

The infant was well and had no jaundice. Studies of cord blood: haemoglobin 14.9 g/100 ml, PCV 50%, and MCHC 29.8%. White cell and platelet counts were normal. The peripheral blood film showed anisocytosis, mild hypochromasia, occasional microcytosis, elliptocytosis and target cells. Haemoglobin H inclusions were absent. Haemoglobin F containing cells were abundant (79%), and 81 / of alkali-resistant haemoglobin was present in the red cell haemolysate. Haemoglobin electrophoresis revealed haemoglobin F and A, and mere traces of HbA_2 and non-haem protein; there was no haemoglobin Bart' or HbH. Serum iron, folate levels and red cell G6PD activity were within normal limits for the newborn. Blood group was AB, rhesus-positive. Total serum bilirubin was 7.2 mg/100 ml.

Postpartum maternal haemoglobin was 9.1 g/100 ml. Both mother and baby progressed well. There was no neonatal jaundice. Both were discharged well on 26-7-75. At postnatal visit, 2 months later, on 24-9-75, both mother and baby were well. Maternal haemoglobin was 8.2 g/100 ml. The other haematological indices showed no change from the initial examinations.

Discussion

The clinical symptomatology in HbH disease is variable. Patients are frequently jaundiced, with hepatosplenomegaly [5 10 13], but our patient did not show these features. Patients also have a mild to moderate chronic haemolytic anaemia with haemoglobin values between 7 and 10 g/100 ml and reticulocytosis up to 5%/ these were present in our patient, together with the usual thalassaemic changes in red cell morphology [2, 5 10, 13]

The percentage of HbH inclusions (62%/) in our patient falls within the reported range of 4–100%/ [10, 13] The reported HbH content of the haemolysate upon electrophoresis varies from 2.5 to 40%/ [2, 5 10] and in our patient, it was 8%. The proportion of red cells affected, and the amount per cell is variable and likely to affect the viability of the cells and degree of haemolysis.

Pre-eclampsia and secondary folate deficiency may occur during pregnancy in patients with HbH disease [5 12] These were not present in our patient. She was nevertheless given folic acid supplements in view of the increased demands of both pregnancy and haemolytic anaemia. Some patients may need multiple blood transfusions [5], but this aspect of the management of chronic haemolytic anaemia must be approached with caution in pregnancy and parturition, as it may precipitate circulatory

failure or marrow depression Splenectomy in the management of HbH disease, has not met with uniformly good results, but some workers have reported clinical improvement [9 12, 13] There was no indication for blood transfusion or splenectomy in our patient.

Oxidant drugs like sulphonamides, nitrofurantoins and salicylates should be avoided in therapy as these may cause the HbH to undergo intravital precipitation in the red cells [5 12, 13] If sulphonamides or nitrofurantoin are required in the treatment of urinary tract infection in the pregnant subject with HbH disease, they must be administered with caution and alternative therapy used where possible.

Genetic evidence suggests that HbH disease results from the interaction of two α-genes, although whether allelic, closely linked or segregating independently it is still not clear [7 14] It is also suggested that three abnormal genes may be involved and HbH disease results when two are present, severity and symptoms depending on the type of gene combination [8] or that, from one to four genes of the α-thalassaemic type may be involved with progressive effects – minimal with one, more pronounced with two HbH disease with three, and Bart s hydrops with all four [6] The case reported here showed no slow-moving Hb fraction and is likely to be of the α-thalassaemia 1 2 doubly-heterozygous type, although there are no family studies to support this The findings on the blood of the patient s husband and baby also do not completely exclude the presence of a minimally expressed α-thalassaemia trait in either

The parent-child transmission of HbH disease has been found to be as high as 21% in Thailand [12] and requires that the other parent also carries an α-thalassaemia gene. If this is an α-thalassaemia 1 gene, the possibility of homozygous Bart s hydrops fetalis also arises in some offspring, with fetal or neonatal loss this occurred in one pregnancy of the case reported by WHITE and JONES [15] It is evident that family studies are important in pregnancies occurring in families where any of the manifestations of α-thalassaemia occur

The pregnant woman with HbH disease will have HbH with high oxygen affinity often together with some Bart s haemoglobin, but these single-chain tetramers do not interact with the larger amounts of HbA present in the red cells, and the normal oxygen dissociation curve is not changed over the physiological range [1] marked disturbance of the relationship between oxygen affinities in maternal and fetal blood would not be expected

It is thus evident that, although the general course of pregnancy asso-

ciated with HbH disease appears favourable, the genetic and haematological considerations may operate to complicate the situation for both mother and child in individual families, and requires full evaluation.

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Cytogenetic Studies in Acute Leukaemias. Prognostic Implications of Chromosome Imbalances

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Key Words. Acute leukaemias · Cytogenetic studies · Giemsa banding · Chromosomal polymorphism · Specific chromosomal abnormalities · Prognosis

Abstract. Consistent cytogenetic abnormalities have been detected in the bone marrow cells of 19 out of 33 patients (57.57%) with a recent diagnosis of acute leukaemia. Chromosome imbalances were apparently non-random, chromosomes 8, 17, 20 and 21 being more frequently involved. The median survival in the patients' group with abnormal metaphases was 55 vs. 210 days in patients with only normal metaphases. In the former group complete remission was obtained in 2 of 3 ALL patients and in 4 of 16 ANLL patients. Major karyotypic abnormalities were consistently found in 5 subjects with EL. Peripheral blood culture lymphocytes showed a 9qh polymorphism in 2 of 35 patients and sporadic or consistent chromosome abnormalities in 6.

Cytogenetic analyses are proved to be a valuable diagnostic and prognostic aid in many cases of leukaemia. The detection of abnormal karyotypes in patients with haematological or clinical signs consistent with a diagnosis of acute leukaemia allows the clinician to reach an earlier correct diagnosis and to start therapy sooner than it would be currently possible if cytogenetic informations were not available [13, 14]. The use of the banding techniques in the study of leukaemic patients has improved the current status of knowledge about the disease; furthermore, at least in some types of acute leukaemia (AL), the cytogenetic status gives useful indications concerning both the clinical course and the response to treatment [7]. Thus, the banding techniques appear to be adequate to investigate several aspects related with onset, evolution and progression of leukaemias.

The present investigation concerns the results of chromosome analyses in 51 successive untreated patients with a recent diagnosis of AL admitted to the Department of Haematology University of Rome, from April 1975 to January 1976 The main purposes of the investigation were to test the non-randomness of the distribution of chromosomes abnormalities and their prognostic significance the frequency of chromosome variants, the frequency and types of abnormal karyotypes from cultured leucocytes.

Material and Methods

Cytogenetic analyses were carried out in 51 leukaemic patients at their first hospitalization, prior to any therapeutic trial (table I). In 33 cases chromosome preparations were obtained from bone marrow aspirates. After 2 h incubation at 37 °C in the presence of colchicine, aspirates were treated with KCl 0.075 M hypotonic solution for 10 min, and fixed with Carnoy's solution before squashing. In 35 cases chromosome analyses were performed on short-term peripheral blood cultures, according to MOORHEAD *et al.* [11] Slides from both bone marrow and peripheral blood were flame dried and stained with Giemsa for routine analysis. G and C banding techniques were applied to slides from 7 to 30 days old. G banding was obtained according to the following procedure: preparations were immersed for 60–150 min at 60 °C in solution of Na-citrat 0.9% (1 part) and NaCl 1.8% (1 part), rinsed in running tap water and stained for 7 min in freshly prepared 5% Giemsa solution in NaCl 0.9%. C banding was done according to the method of SALAMANCA and ARMENDARES [17]. Not less than 10 cells from each patient were examined by conventional techniques and as many as possible by G and C banding (range 3–20 cells). Histochemistry data were available in all patients. The cases were classified as acute lymphoid leukaemia (ALL), acute myelocytic leukaemia (AML), acute myelomonocytic leukaemia (AMML), and erythroleukaemia (EL) according to criteria described by HAYHOE [8], HAYHOE and CAWLEY [9] and BLOOMFIELD and BRUNNING [2]. Evaluation of clinical course and response to therapy was done following criteria similar to those reported by GOLOMB *et al.* [7].

Results

The cytogenetic analyses from direct bone marrow preparations in the 33 patients with acute leukaemias have shown the presence of abnormal karyotypes in 19 cases. Details are summarized in table II. Two patients with ALL (cases 1 and 2) in which 7 and 10% of metaphases were found to be numerically abnormal had complete remission (CR), still lasting 19 months later In a third ALL individual with 25% of hypoploid

metaphases, the most consistent finding being the loss of an E group chromosome death occurred before reaching remission 16 out of 30 acute non lymphatic leukaemia patients (ANLL) displayed abnormal karyotypes in a proportion of cells ranging between 5 and 100%. CR was obtained only in 4 cases in 3 of them, 2 AML and 1 AMML, the percentage of abnormal cells was less than 10%, while in the 4th case (AML), they were 73% Case No 8 (AML) had partial remission (survival 15 months) despite the presence of abnormal clones in 100% of examined cells. However it is noteworthy that the Y chromosome was lacking in all metaphases. The abnormality was associated with trisomy 8 in 10% of cells In the other 3 AML patients in which the proportion of metaphases with aberrant karyotypes ranged between 60 and 100%, death occurred before remission Despite the different percentage of abnormal clones found in the other 3 subjects with AMML (5 33 and 90%, respectively) and the involvement of different chromosomes, all deceased before remission It is of interest that 4 out of 7 AMML patients with only normal karyotypes are still in CR from 13 to 19 months, while the other 3 cases deceased in the 7th 8th and 9th month, respectively

The 5 patients with acute EL showed a common pattern of cytogenetic abnormalities presence of abnormal clones in 100% of cells, prevalence of hypodiploid clones, high proportion of chromosome instability and multiple rearranged cell lines, presence of rings. These chromosome imbalances resulted in a very poor prognosis (median survival <1 month) none of these cases having reached remission before death. A 17 year-old EL patient with only 46 XX normal cells is now in the 13th month of partial response to therapy

The results of chromosome investigations of PHA-stimulated peripheral blood cultured leucocytes of 35 leukaemic patients are summarized in table III A high frequency of normal karyotypes has been demonstrated in all cases. However in 6 of them occasional abnormal cells were observed Most of these abnormalities occurred in individual cells, involving different chromosomes. In three cases, frequency or type of chromosome abnormalities appeared consistent and non random In one ALL patient with Louis Bar syndrome, 12% of examined metaphases showed gaps and/or chromosomal and chromatid breaks in one ANNL patient 3 cells with trisomy 8 were found in another ANLL subject the same marker chromosome detected on bone marrow preparations was demonstrated in 4 metaphases and divergent karyotypes in 4 additional cells.

Major chromosome variants, as derived by C banding constitutive heterochromatin studies, were observed in two individuals. One patient in the ANLL group and one ALL patient showed a 9qh polymorphism. The ANLL patient (case 6 in table II) deceased before remission.

Discussion

The present series consists of all the consecutive untreated acute leukaemic patients admitted to the Department of Haematology University of Rome, in a period of 10 months. However the sample is biased in favour of individuals in which the metaphases were of good quality In fact, only the cases in which the results of chromosome preparations and chromosome banding were of such good quality as to permit the exact identification of karyotype have been included. Abnormal metaphases have been found on direct bone marrow preparations in 19 out of 33 patients (57.5%) The sample of patients with abnormal karyotype demonstrates an apparently non-random gain or loss of chromosomes. Trisomies 8 and 21 were detected in five subjects trisomies 16, 17 and 19 in two individuals, in singular cases trisomies 9 20 and 22 have been observed. Chromosomal loss involved No 21 in four cases, No. 7 in three cases and No. 8, 10 and 17 in two cases each, chromosomes 18 and 20 were lacking in individual patients. Therefore, our data are consistent with the preferential involvement of specific chromosome in imbalances, which in the present series were No 8, 17 20 and 21

The correlation between chromosomal pattern and prognosis is a further argument for debate. While SAKURAI and SANDBERG [15] and GOLOMB *et al* [7] have found worse prognosis in subjects with abnormal karyotypes than in those with normal ones, FORD *et al.* [6] HART *et al.* [10] and FITZGERALD *et al.* [5] have denied the possibility to correlate the prognosis with the cytogenetic findings. Our data indicate a median survival of 55 days in the 16 ANLL patients with abnormal karyotypes and of 210 days in the 14 ANLL patients with normal karyotypes. However it is worthy to note that 5 patients in the latter group are still in remission from 13 to 19 months, since their first diagnosis.

Personal experience does not allow to conclude for the existence of consistent different prognostic significance related with the involvement of different chromosomes in ANNL patients. TRUJILLO *et al.* [18] have pointed out significant median survival differences between AL adults

metaphases, the most consistent finding being the loss of an E group chromosome death occurred before reaching remission. 16 out of 30 acute non lymphatic leukaemia patients (ANLL) displayed abnormal karyotypes in a proportion of cells ranging between 5 and 100%. CR was obtained only in 4 cases in 3 of them, 2 AML and 1 AMML, the percentage of abnormal cells was less than 10%, while in the 4th case (AML), they were 73%. Case No 8 (AML) had partial remission (survival 15 months) despite the presence of abnormal clones in 100% of examined cells. However it is noteworthy that the Y chromosome was lacking in all metaphases. The abnormality was associated with trisomy 8 in 10% of cells In the other 3 AML patients in which the proportion of metaphases with aberrant karyotypes ranged between 60 and 100%, death occurred before remission. Despite the different percentage of abnormal clones found in the other 3 subjects with AMML (5 33 and 90%, respectively) and the involvement of different chromosomes, all deceased before remission It is of interest that 4 out of 7 AMML patients with only normal karyotypes are still in CR from 13 to 19 months, while the other 3 cases deceased in the 7th 8th and 9th month respectively

The 5 patients with acute EL showed a common pattern of cytogenetic abnormalities presence of abnormal clones in 100% of cells, prevalence of hypodiploid clones, high proportion of chromosome instability and multiple rearranged cell lines, presence of rings. These chromosome imbalances resulted in a very poor prognosis (median survival <1 month), none of these cases having reached remission before death. A 17 year-old EL patient with only 46 XX normal cells is now in the 13th month of partial response to therapy

The results of chromosome investigations of PHA-stimulated peripheral blood cultured leucocytes of 35 leukaemic patients are summarized in table III A high frequency of normal karyotypes has been demonstrated in all cases. However in 6 of them occasional abnormal cells were observed Most of these abnormalities occurred in individual cells, involving different chromosomes. In three cases, frequency or type of chromosome abnormalities appeared consistent and non-random. In one ALL patient with Louis-Bar syndrome, 12% of examined metaphases showed gaps and/or chromosomal and chromatid breaks in one ANNL patient 3 cells with trisomy 8 were found in another ANLL subject the same marker chromosome detected on bone marrow preparations was demonstrated in 4 metaphases and divergent karyotypes in 4 additional cells.

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with –C, +D +E, –G karyotypes (80 weeks on average), +C patients (68 weeks) and –C individuals (15 weeks) respectively However according to the observations of GOLOMB *et al* [7] who have reported median survival of 6 months and 1 month respectively the present series of patients does not support the conclusions of TRUJILLO *et al.* [18] In agreement with our previous data [1] and with those reported by SAKURAI and SANDBERG [16] it is suggested that the presence of ring chromosomes is associated with a very poor prognosis (median survival <1 month) Furthermore, it is confirmed that this rare chromosome aberration is a rather common pattern of EL, most often affecting elderly patients.

PHA-stimulated peripheral blood cultures have shown interesting results concerning both the frequency of chromosomal polymorphism and frequency of abnormal karyotypes Experience from different laboratories [3 4] is consistent with an increased frequency of major chromosomal variants in leukaemic patients. In the present investigation, 2 subjects have shown a 9qh chromosome. This figure (2.6%) is higher than it is expected in the general population (about 1‰) although exact data concerning the population of this country (Italy) are still lacking. Further studies are needed to support the above evidence derived from our patient sample. According to the results of studies of families in which a 9qh polymorphism is segregated which indicate a significant increase of risk of non-disjunction leading to major chromosome aberrations in offsprings [12] an excess of heterochromatin could have a relevant role in the karyotype s clonal evolution in these patients, with a consequently worse prognosis for the disease.

In 6 out of 35 patients sporadic or consistent chromosomal aberrations have been found in preparations obtained from PHA-stimulated leucocytes. In most, but not in all cases, the types of aneuploidies do correspond to those found on direct bone marrow preparations. Chromosomes 8 and 21 appear to be more consistently involved as compared with other chromosomes. These findings are in agreement with other observations [6] and demonstrate, at least in a proportion of patients, the usefulness of PHA-cultured leucocyte studies.

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Methods

Coagulation studies. The celite activated partial thromboplastin time (APTT) and the prothrombin time (PT) were measured with commercial Warner Chilcott reagent.

Factors II, V and X were measured with commercial reagents of Behringwerke. Factors VIII, IX, XI and XII were measured in plasma by modified celite PTT assay using congenitally deficient substrates [3]. Factor XI was also assayed in celite eluate prepared according to NOSSEL [16]

Test for circulating anticoagulants were performed measuring APTT and PT of different mixtures of normal and patient plasma [3]. Inhibitory activity against factor XI was assayed as described by CASTALDI and NILSSON [6]. The inhibitory activity against factor XI was also assayed according to NIEMETZ and NOSSEL [15] in patient globulin fraction as prepared by KARPATKIN *et al.* [11].

Platelet investigation. The bleeding time was determined according to DUKE [3], platelet count was performed on whole blood [2]. Platelet aggregation was estimated by the method of BORN [3–5] using as aggregating agents ADP (Boehringer) and collagen (Horm). Antiplatelet antibodies were estimated by the antiglobulin consumption test according to STEFFEN [19], by release of platelet factor 3 according to KARPATKIN *et al.* [11], and by serotonin release according to HIRSCHMAN and SHULMAN [8].

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Case Report

Patient N. B., 40-year-old woman, was admitted to the Policlinico Gemelli of Rome on February 2, 1976. Since January 1975 she developed joint pains with swelling, and for the last month petechiae have appeared on her legs. On physical examination, few small ecchymoses were present on the limbs and the abdomen; many petechiae were seen on both lower extremities. Physical examination of the lung, the heart, the liver and the spleen was normal.

The hemoglobin value was 10.2 g/ml with hematocrit of 30%. The leukocyte count was 3,500/mm³ with normal differential count. Platelet count was 20,000/mm³. Sternal bone marrow showed nonspecific changes: the megakaryocytes were numerous. Screening coagulation studies showed PT of 13 sec and an APTT of 54 sec (table I).

Serum values for urea nitrogen, creatinine, bilirubin, alkaline phosphatase and glutamic oxaloacetic transaminase were normal. Urinalysis was negative. Plasma protein analysis with electrophoresis and immunoelectrophoresis was normal. Tests for thyroid function were normal, but the antithyroglobulin test was positive. Direct and indirect Coombs tests were negative, agglutination with enzyme-treated cells was positive. Systemic lupus erythematosus was diagnosed on the basis of the posi-

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Circulating Anticoagulant against Factor XI and Thrombocytopenia with Platelet Aggregation Inhibition in Systemic Lupus Erythematosus

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Key Words. Antiplatelet antibodies · Circulating anticoagulant · Factor XI · Platelet aggregation · Systemic lupus erythematosus

Abstract. In a patient with systemic lupus erythematosus, anticoagulant activity directed against factor XI was found together with thrombocytopenia. In the serum globulin fraction, antiplatelet antibodies and an activity-inhibiting platelet aggregation could also be found. A possible correlation between the inhibition of platelet aggregation and the anticoagulant activity directed against factor XI is discussed.

Circulating anticoagulant sometimes occurs in patients with systemic lupus erythematosus (SLE) [1, 4, 6, 7, 12–14, 17, 18]. The antibodies are mostly directed against the prothrombin activator complex, against factor VIII and against factor IX [1, 4, 9, 13, 14, 17, 18]. In such patients severe bleeding symptoms occur; bleeding in SLE may also be related to thrombocytopenia [1, 4], which almost always is associated with the presence of antiplatelet antibodies [8, 11]. However, some cases with anticoagulants against factor XI [4, 12] and factor XII [4, 6, 7] have also been reported.

We recently had the opportunity of studying an anticoagulant directed against factor XI in a patient with an autoimmune disorder of the SLE type. In that case there was also thrombocytopenia with the presence of antiplatelet antibodies inhibiting platelet aggregation.

Methods

Coagulation studies. The celite activated partial thromboplastin time (APTT) and the prothrombin time (PT) were measured with commercial Warner Chilcott reagent.

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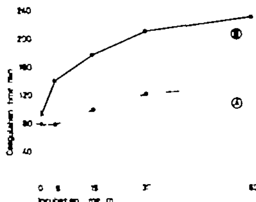


Fig 1 Inhibition test of contact product. *A* Normal globulin fraction. *B* Patient globulin fraction.

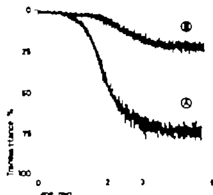


Fig 2 Inhibition of platelet aggregation by collagen *A* Normal globulin fraction. *B* Patient' globulin fraction.

Discussion

The hemorrhagic diathesis in SLE can be due to anticoagulants and/or to thrombocytopenia [1 4 6 7 9 11–13 17 18] Generally the anticoagulants are directed against prothrombin activator complexes, factors VIII and IX [9 13 14 17 18] and very rarely against factors XI [4–12] and XII [6, 7]

Table I Hemostatic studies

	Patient	Normal range
Bleeding time – Duke, min	1	3–4
Activated thromboplastin time, sec	56	25–40
One-stage prothrombin time sec	13	12 13.5
Platelet number/µl	30,000	200,000–400,000
Factor II %	90	80–120
Factor V	110	50–150
Factors VII+X, %	90	80–120
Factor X, %	95	60–140
Factor VIII %	90	60–160
Factor IX,	100	60–160
Factor XI %	10	60–160
Factor XII	80	60–160
Fibrinogen, mg%/ml	350	200–400

Mixture 1/1 of normal and pathological plasma incubated 30 min APTT 64 sec (control, 40 sec) residual factor XI 15 % (control 50%).

tive lupus erythematosus (LE) cell preparation and the positive antinuclear factor (ANF) test.

The patient was treated with methylprednisone, 80 mg daily and her general conditions improved. APTT returned to normal after a few days. Subsequently the platelet count also returned to normal value.

Coagulation stud s Hemostatic studies are shown in table I The PT was normal, APTT was prolonged, and addition of the patient's plasma to normal plasma prolonged the APTT after incubation at 37 C for 30 min. These findings suggested an anticoagulant interfering with intrinsic clotting. The values found for factors VIII, IX and XII were normal. Factor XI in the patient s plasma as assayed on factor XI-deficient plasma as a test base was 10%. Moreover the contact product of the patient accelerated clotti g of noncontacted plasma to the same degree as normal contact product (Nossel test). The patient s celite eluate corrected the APTT of factor XI-deficient plasma, normally But when equal volumes of normal plasma and patients plasma were incubated at 37 C for 30 min factor XI was consumed Globulin fraction of patient serum also inhibited the accelerating effect of the contact product on coagulation of noncontacted plasma (fig. 1)

Platelet investigation The platelet were reduced in number Antiplatelet antibodies were found with the globulin consumption method, and the serotonin release method, but not with the PF3 release method. The patient s globulin fraction inhibited platelet aggregation by collagen and ADP of normal platelet-rich plasma (fig. 2).

Fibrinoly tic studies. No abnormality in the f brinolytic system was detected.

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Our case is interesting because in the same subject an anticoagulant activity and thrombocytopenia were found. Furthermore the anticoagulant activity was directed against factor XI which is also found in platelets washed repeatedly [16] The patient s globulin fraction inhibited factor XI activity and platelet aggregation A tenable hypothesis could be that the same antibody inhibited the factor XI and the platelet aggregation [10] However against this interpretation the patient s globulin fraction also inhibited the platelet aggregation of a subject affected by a defect of factor XI and after treatment anticoagulant activity against factor XI disappeared before the activity inhibiting platelet aggregation

As in one of the cases described by CASTRO *et al* [43] specific assay for factor XI gave a low result in the plasma but not in the celite eluate. Nevertheless, the patient s globulin fraction inhibited the accelerating effect of the contact product on coagulation of noncontacted plasma. This fact suggests that the inhibitor is directed against factor XIa. Moreover it is loosely bound to factor XI therefore easily eluted from the contact product.

A comment must be made about the methods for detecting antiplatelet antibodies in this case Among the tests used, only the Karpatkin test was negative. Certainly in the presence of anticoagulants, the Karpatkin test is not a valuable technique because the anticoagulant activity conceals the accelerating effect on plasma coagulation of PF3 release produced by antiplatelet antibodies

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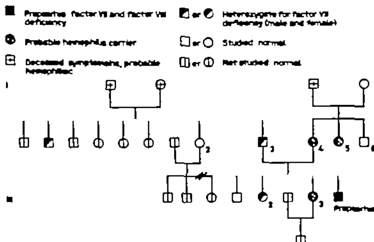


Fig 1 Family tree. The father paternal uncle and sister of our propositus are heterozygotes for factor VII deficiency The mother maternal aunt and sister are probably hemophilia carriers Unfortunately the other paternal relatives of our propositus were not availabl for study On statistical basis one may expect for sure that other heterozygotes for factor VII deficiency are present among them. The deceased probable hemophiliac is the maternal grandfather of our propositus.

combined factor VII and factor VIII defect was present [8] The defect appeared to be transmitted as an autosomal dominant trait and was interpreted to be the result of a common defect in one gene or in a system of genes involved in the activation of factor VII and factor VIII. On the same occasion we had postulated, on the basis of what already demonstrated for combined factor V and factor VIII deficiency the possibility that two types of combined factor VII and factor VIII deficiency might exist. Should that be true then one could speculate that the family previously described by us could represent an example of type II disease.

The present report deals with a case of combined factor VII and factor VIII deficiency due to the casual association of heterozygosis for factor VII deficiency and hemophilia A.

Material and Methods

Methods have been discussed in detail in previous recent papers and need not be repeated herein [3–5, 7 9].

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Combined Factor VII and Factor VIII Deficiency Due to a Casual Association of Heterozygosis for Factor VII Deficiency and Hemophilia A[1]

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Key Words. Factor VII · Factor VIII · Combined deficiency · Heterozygosis · Hemophilia A

Abstract. A patient with combined factor VII and factor VIII deficiency is discussed. The propositus is a 21-year-old male who presented a mild bleeding tendency. The patient appears to be a hemophilia and at the same time heterozygote for factor VII deficiency. This conclusion is based on the fact that heterozygosis for factor VII deficiency was present in the father and in other relatives of the paternal side. On the contrary, no factor VII deficiency was present in the maternal side of the family. However, the maternal grandfather was known to have been a bleeder and the propositus' mother, his sister and his aunt had low-normal factor VIII levels and were probably hemophilia A carriers. This type of combined factor VII and factor VIII deficiency appears to be due to the casual association of two independently segregating defects.

Combined congenital disorders of clotting factors are rare clinical entities. The only condition which seems already well proved is the combined deficiency of factor V and factor VIII [5, 9–13, 15]. As a matter of fact, two distinct types of combined factor V and factor VIII deficiencies appear to exist [9, 10]. Type I is due to a casual association of heterozygosis for factor V and hemophilia A. Type II is probably due to the lack of malfunctioning of an autosomal gene or of a system of autosomal genes involved in factor V and factor VIII activation. In both instances, factor VIII associated antigen was found to be normal [6, 9, 10, 16].

We have recently described seven members of a family in whom a

[1] This study was supported in part by a grant from the CNR (grant CT 75.00992.04), Rome, and by a grant from the Anna Villa Rusconi Foundation, Varese, Italy.

Table I. Coagulation study in our propositus

Test	Result	Normal values
Bleeding time	3 min 30 sec	<5 min
Tourniquet	negative	negative
Platelet count	200,000	150,000–350,000
Clot retraction	complete after 6 h	complete after 10 h
Platelet adhesion to glass	35 %	30–60 %
Platelet aggregation (ristocetin)	65 %	72 ± 12.5 %
PTT	76,3 sec	32 – 42 sec
PT	16.8 sec	13 – 14 sec
TT	19 sec	18 – 25 sec
TGT	32 sec in 8 min	16 sec in 6–8 min
Fibrinogen	370 mg	250–450 mg
Factors II, V IX, X, XI, XII	normal	60–160 %
Factor XIII (clot solubility in 5 % urea)	clot insoluble	clot insoluble
Factor VII	45 %	80–120 %
Factor VIII	3 %	60–160 %
Euglobulin lysis time	15 h	10–30 h

Table II. PTT cross-correction studies in the propositus

Mixtures (equal parts)	PTT sec	PTT (sec) reference plasma
Patient plasma	76 6	
Patient plasma + normal serum	78 2	
Patient plasma + adsorbed normal plasma	42.1	
Patient plasma + hemophilia A plasma	91 5	110
Patient plasma + plasma of patient with combined factor VII and factor VIII deficiency	68 6	52.5

Results

The results of coagulation study are summarized in tables I and II. Platelet and vascular tests were all within normal limits and there was no fibrinogen or fibrinolytic activity changes. Plasmatic tests showed a mod erate prolongation of the PTT together with a mild prolongation of the PT On the contrary thrombin time was normal. Specific assay demon

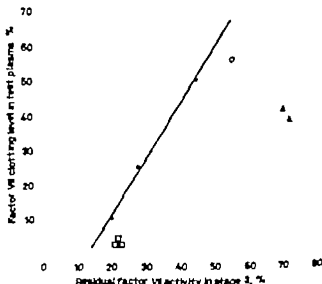


Fig 2 Relationship of factor VII clotting activity and residual factor VII activity in the antibody neutralization assay (stage 3) in the propositus and his father (open circles) The open squares refer to 3 patients with severe factor VII deficiency whereas the dark triangles refer to 2 patients with combined factor VII and factor VIII deficiency belonging to another family (type II disease) The dots and the best fit line represent the calibration curve as obtained by serial dilutions of pooled normal plasma. Note that the factor VII antigen appears to be proportional to the factor VII activity in our propositus whereas it results to be definitely higher in the case of combined factor VII and factor VIII of type II

Case Report

The propositus is a 21 year-old male who was sent to us for further study in April 1976 after a routine coagulation evaluation showed the presence of a prolonged PTT Family history was positive for bleeding disorders in the sense that the maternal grandfather of our propositus was known to be a 'bleeder' and had died because of massive bleeding from a duodenal ulcer (fig. 1). Our propositus was first noted to have occasional epistaxis or easy bruising in childhood. However bleeding manifestations have always been mild and have drawn no particular attention. In June 1975 the patient was admitted to a local hospital because of pain in the right flank area after a minor trauma On that occasion a persistent microscopic hematuria was noted but routine renal function studies revealed no abnormality In late 1975 the patient had a car accident which caused the appearance of a hematoma of the right groin area. The hematoma subsided after prolonged bed rest and one unit of fresh blood. In March 1976 the patient was admitted to a Nephrology Department for an evaluation of the persistent hematuria and on this occasion a routine coagulation study showed, on repeated assays, a prolonged PTT At the time of study in Padua there were no bleeding manifestations.

Table IV Tentative classification of combined deficiency of factor VII and factor VIII in two groups

Condition	Frequence	Hereditary pattern	Factor VII activity	Factor VII antigen	Factor VIII activity	Factor VIII antigen
Type I pure association type	?	independent segregation	intermediate	intermediate	low or very low (lower than factor VII)	normal
Type II, common gene abnormality	?	autosomal dominant	low (similar to factor VIII level)	normal	low (similar to factor VII level)	normal

strated that factor VII was 45 / of normal whereas factor VIII was 3 / of normal. The other clotting factors were within normal limits. No factor VII or factor VIII inhibitors were present. Factor VIII antigen was normal whereas factor VII protein, as evaluated in a neutralization test, was equivalent to the clotting counterpart (fig. 2) The electrophoretic mobility of factor VIII in the bidimensional electrophoretic system is similar to that presented by normal factor VIII (fig. 3). Platelet and vascular tests including platelet adhesiveness and aggregation were within normal limits. The study of family members revealed that the father and one sister had slightly reduced factor VII levels (table III) The mother of the propositus, his maternal aunt and one of her sisters showed a slightly decreased or low normal factor VIII level and normal factor VIII antigen. They were considered as probable hemophilia carriers.

Discussion

The coagulation study revealed the presence of a moderate first-stage defect together with a mild second-stage defect. Specific assays revealed the presence of moderately decreased factor VIII activity and of mildly decreased factor VII activity This is compatible with the diagnosis of combined factor VII and factor VIII deficiency The only acceptable explanation was to admit that propositus is a hemophilic and at the same time heterozygote for factor VII deficiency The laboratory findings are all con-

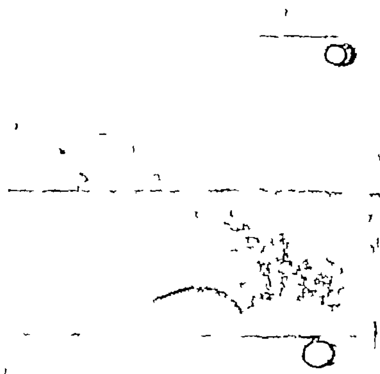


Fig 3 Bidimensional immunoelectrophoresis of normal plasma (bottom) and propositus plasma (top). The electrophoretic mobility of factor VIII antigen appears to be the same in both instances.

Table III Factor VII and factor VIII in the propositus, parents and siblings

Patient (position in family tree)	Factor VII activity	Factor VII neutralization test	Factor VIII activity	Factor VIII antigen (Laurell method)
II_3, father	48	55	1[illegible]0	160
II_4, mother	110	95	50	90
III_1, brother	100	100	96	110
II_1, sister	55	50	100	100
II_2, sister	96	100	55	100
Propositus	45	50	3	90
Normal values	80–120	70–130	60–160	60–160

Table IV Tentative classification of combined deficiency of factor VII and factor VIII in two groups

Condition	Frequence	Hereditary pattern	Factor VII activity	Factor VII antigen	Factor VIII activity	Factor VIII antigen
Type I pure association type	?	independent segregation	intermediate	intermediate	low or very low (lower than factor VII)	normal
Type II, common gene abnormality	?	autosomal dominant	low (similar to factor VIII level)	normal	low (similar to factor VII level)	normal

strated that factor VII was 45 % of normal whereas factor VIII was 3% of normal. The other clotting factors were within normal limits. No factor VII or factor VIII inhibitors were present. Factor VIII antigen was normal whereas factor VII protein, as evaluated in a neutralization test, was equivalent to the clotting counterpart (fig. 2). The electrophoretic mobility of factor VIII in the bidimensional electrophoretic system is similar to that presented by normal factor VIII (fig. 3). Platelet and vascular tests including platelet adhesiveness and aggregation were within normal limits. The study of family members revealed that the father and one sister had slightly reduced factor VII levels (table III). The mother of the propositus, his maternal aunt and one of her sisters showed a slightly decreased or low normal factor VIII level and normal factor VIII antigen. They were considered as probable hemophilia carriers.

Discussion

The coagulation study revealed the presence of a moderate first-stage defect together with a mild second-stage defect. Specific assays revealed the presence of moderately decreased factor VIII activity and of mildly decreased factor VII activity. This is compatible with the diagnosis of combined factor VII and factor VIII deficiency. The only acceptable explanation was to admit that propositus is a hemophilic and at the same time heterozygote for factor VII deficiency. The laboratory findings are all con-

sistent with this assumption The mother an aunt and a sister are probably hemophilia carriers The father of the propositus, a paternal uncle and a sister are probably heterozygotes for factor VII deficiency In this regard it is worth noting that the factor VII levels found in the propositus and some of his relatives are higher than those found in the family previously studied by us. Furthermore, in the previous family the factor VII levels were similar to the factor VIII levels, namely to 25–30% [8] This interpretation is confirmed by the neutralization studies which showed that the propositus and some of his relatives have moderately reduced antigen levels, comparable to the clotting counterparts. On the contrary in the previous family there was a clear discrepancy between factor VII activity and factor VII antigen Finally the hereditary pattern is different. It appears to be autosomal dominant in the former whereas it represents the independent segregation of two defects in the latter In agreement with what had already been proposed by us for combined factor V and factor VIII deficiency we think two types of combined factor VII and factor VIII exist (table IV) The first type dealt with by us in the present paper is the result of a casual association of heterozygosis for factor VII deficiency with hemophilia A We propose to call this condition type I The second type of deficiency (type II) is characterized by a defect in an autosomal gene or a system of genes involved in factor VII and factor VII activation

The families studied by GASTON *et al* [2] and by CONSTANDOULAKIS [1] as noted in a previous paper [8] are in full agreement with the classification herein proposed The first family may in fact belong to type I disease, whereas the second one seems to behave to type II Another consideration seems also justified So far no association of factor II or factor X with factor VIII deficiency has been described. The fact that factor II and factor X deficiencies are much rarer coagulation disorders as compared with factor V and factor VII defects, may explain the phenomenon at least for type I diseases, the pure association type

The possibility that factor VIII activation may be dependent on a gene system which is also involved with the activation of factor VII and V but not of factor II and X seems remote but cannot be completely ruled out These studies on combined defects emphasize the importance of a careful evaluation of routine screening tests, namely PTT and PT A mild prolongation of the prothrombin time in a hemophiliac should not be considered as a vagary of the test but the expression of a possible concomitant defect of one of the factors influencing the test. In a series of about 400

hemophilia A and B patients who were studied by us in the past 9 years we found a constant prolongation of 1,5–3 sec or more in about 25 cases. In hemophilia A patients both associated factor V and factor VII defi ciencies were found to be present. On the contrary in hemophilia B only a variable, concomitant factor VII defect was found. Furthermore, the ex istence of combined factor V or factor VII and factor VIII defects indi cate the complexities of factor VIII activation.

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R. Biggs: Human Blood Coagulation, Haemostasis and Thrombosis. 2nd ed. Blackwell, Oxford 1976. XXVIII + 770 pp. £ 15.00. ISBN 0-632-00921-7

At present it is difficult if not impossible to cover sufficiently the whole field of blood coagulation, haemostasis and thrombosis in one volume.

T certain extent the editor and co-authors of this new book and of the first edition have made an exception. For those who are dealing in particular with practical problems of blood coagulation and bleeding disorders this book will be welcome manual.

A disputable point of the book is that in contrast t chapters dealing with blood coagulation, topics on platelet function and (patho-)physiology and thrombosis are not comprehensively covered and may need further extension. In this respect, the more recent developments of the biochemical aspects of the hemostatic mechanism, including the mechanism f activation of the intrinsic and extrinsic coagulation factors, fibrinogen and the mode of interaction of various coagulation inhibitors have not been treated extensively as well. One might wonder however whether an extensive review of recent progress made in these still rapidly developing fields is really necessary in book of this type.

The second edition as the original book deals mainly with the established aspects of blood coagulation and hemostasis and it is not surprising to find that this new edition is not revised extensively in this respect. It should be noted that the final production of the book has encountered some unfortunat delay. Therefore, some recent information could not be included. The chapter on therapeutic concentrates, for example, is extended but information on the use of activated prothrombin complex concentrates for the management f hemophiliacs with circulating inhibitors is not given.

Some general chapters on coagulation, the hemostatic process and thrombosis and also the technical appendices are still in their original form or are only slightly revised.

Although the title of the book does not seem to be fully covered by its content it can be said that those ho are interested in practical guide for the study of blood coagulation and the management of bleeding disorders this book can be highly recommended.

J. A. v. Mourik, *Amsterdam*

M. R. Parwaresch: The Human Blood Basophil. Origin, Kinetics, Function and Pathology. Springer, Berlin 1976. XI + 235 pp., DM 97. ISBN 3-540-07649-...

I found this book so interesting and well ritten that I have ead it from cover to cover in single day. Parwaresch clearly finds the basophil most fascinating cell and of course he is right. I this monograph he brings together an immense, if scattered and often contradictory literature, sorts and analyses it, supplements it with some notable original experimental ork of his own, and presents us ith

comprehensive and up-to-date account of the basophil cell, its cytology cytochemistry ultrastructure and function in health and disease The original studies of the degranulation and lysis of blood basophils, with liberation of heparin and histamine, which follows the hyperlipidaemia induced by a fatty meal, and the subsequent release of young basophils with cytochemically less mature granules from the bone marrow indicate an important role for basophils in serum lipolysis. The binding of histamine to heparin, itself bound to l poprotein in the basophil granule, and the release of these substances at cell surfaces involved in immune reactions, demonstrate the contribution of basophils, triggered by IgE, to hypersensitivity responses. The author provides new data on basophil cell kinetics, showing that blood basophils have a short life but a high marrow replacement rate His account of basophil involvement in leukaemias and other myeloproliferative processes is well balanced, although the view that basophils and also eosinophils descend from non specific promyelocytes (pp 92, 192, 218) does not take account of recent ultrastructure observations, which suggest that the promyelocytic 'primary granules of eosinophils, basophils and neutrophils are distinct in structure and imply that the common precursor of these lines is an agranular blast cell rather than a granular promyelocyte [e g. CAWLEY and HAYHOE. Ultrastructure of Haemic Cells, Saunders, Philadelphia 1973] This criticism does not affect the conclusions on nosology and interrelationships between the variants of myeloid leukaemia It merely sets divergence from a common path at a more primitive stage before primary granules appear

All in all, this is an excellent monograph, and worthy of strong recommendation. F G J HAYHOE *Cambridge*

J JAENECKE. **Antikoagulantien- und Fibrinolysetherapie.** _ Aufl Thieme, Stuttgart 1976. VI + 184 pp 36 fig., 14 tab DM 14.80 ISBN 3 13-471302-0.

Dieses handliche Taschenbüchlein ist ein würdiger Nachfolger der in eigener Praxis vielfach bewährten ersten Auflage Der didaktische Aufbau, die Tabellen und Abbildungen und die Texteinteilung sind durchwegs übersichtlich und ansprechend In der neuen Auflage sind die jüngsten, d e peroperative Thromboseprophylaxe betreffenden Studien bereits berücksichtigt und auch theoretisch belegt. Insgesamt werden neue Indikationen und Therapieformen (z B. die Anwendung der Thrombozyten-Aggregationshemmers) mit der notwendigen Zurückhaltung diskutiert Allerdings scheint der Autor die Fibrinolysetherapie mit einer nicht mehr allerorts akzeptierten positiven Grundhaltung zu beurteilen. Bedauerlich ist die summarische Behandlung des therapeutischen Bereiches der oralen Antikoagulation (p. 55). Ich hätte mir gerade auf diesem Gebiet n der Neuauflage eine kritische Würdigung der Bestrebungen für eine internationale Standardisierung dieser äusserst komplexen Therapie gewünscht Im methodischen Teil werden nach wie vor recht umständliche (z.T ungenaue) manuelle Methoden vordergründig beschrieben. Abgesehen von diesen Einschränkungen handelt es sich um ein mustergültig gestaltetes, handliches Übersichtswerk, welches dem Fachhämatologen und Allgemeinpraktiker aber auch dem Medizinstudenten und Technischen Assistenten gleichermassen empfohlen werden kann. E. A. BECK, *Bern*

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Acquired Aplastic Anaemia in Adults

I. A Retrospective Analysis of 40 Cases: Single Factors Influencing the Prognosis

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Key Words. Aplastic anaemia · Prognostic parameters

Abstract. In a retrospective analysis of 40 aplastic anaemia patients, an attempt was made to determine prognostic parameters permitting discrimination between short survivors (<6 months) and long-term survivors (>6 months). Short survival proved to be significantly associated with a persistently low reticulocyte index and progressive neutropenia. Other factors such as bone marrow cellularity, HBF level, aetiology or presenting signs were not indicative.

Chromosomal aberrations were found in one third of the cases examined, but none had developed into a leukaemic stage after an observation period of 2–5 years. None of the patients with cytogenetic aberrations showed complete restoration of haematopoiesis, in contrast to several of the patients without chromosomal abnormalities. In this series, drug-associated aplastic anaemia was found more often in the short-survival group. These clinical findings indicate that the term 'aplastic anaemia' covers a heterogenous group of disorders.

Normal blood cell counts are the result of an equilibrium between production and destruction. Pancytopenia, which is due to decreased cell production, is referred to as bone marrow failure. This syndrome is usually associated with several neoplastic and metabolic disorders, e.g. leukaemia, storage diseases, deficiencies, or uraemia. In rare instances none of these disorders can be demonstrated.

This category includes the 'drug-induced', hepatitis-associated, and idiopathic forms of bone marrow failure designated as 'aplastic anaemia'. It should be stressed, however, that a common name does not necessarily mean a common aetiology or pathogenesis.

On this basis, the criteria for the diagnosis aplastic anaemia (table I) are pancytopenia in the peripheral blood and loss of haematopoietic parenchyme in the bone marrow demonstrated cytologically and histologically without evidence of malignancy and with at most a small focal increase of reticulin fibres. The presence of hepatomegaly splenomegaly or lymphadenopathy argues against the diagnosis. Deficiencies, metabolic or storage diseases, and extramedullary haematopoiesis should be excluded. For the present study we selected criteria similar to those in the literature [20 23 32, 46] to permit comparison with the published results

Although this definition includes mild forms of pancytopenia, the majority of the cases seen by the clinician have a much more pronounced degree of bone marrow failure. In general the outlook for the patients is grim The selection of therapeutic measures is hampered by the difficulty of determining the prognosis of a given patient at presentation [13 29 32] THOMAS [42] concluded that in severe aplastic anaemia bone marrow transplantation (BMT) is the therapy of choice [8] On the other hand, many clinicians know a few patients who survived severe pancytopenic periods and recovered spontaneously

The aim of this study was to find clinical and laboratory parameters that can be used to determine the prognosis in aplastic anaemia

Patient Selection

We reviewed the case histories of 43 patients admitted to the Leiden University Hospital between January 1st 1964 and January 1st 1976, and diagnosed as having aplastic anaemia. 3 cases originally classified as aplastic anaemia but retrospectively showing evidence of acute myelogenous leukaemia, were excluded. The remaining

Table I Criteria for the diagnosis aplastic anaemia ✓

Cytopenia Hb < 10 g/dl PMN < 1,500/µl
Pl < 100,000 µl
BM histology and cytology
- Loss of haematopoietic parenchyme
- No malignancy or storage disease
- No extensive fibrosis

No lymphadenopathy or hepatosplenomegaly
No deficiencies or metabolic disease
No evidence of extramedullary haematopoiesis

40 cases were divided into 3 groups (table II). Group I is composed of 11 patients who died within 6 months after diagnosis, and group II of 18 patients who were still alive 6 months after diagnosis. Group III includes all patients given pretreatment for BMT and was formed because this drastic treatment could have reduced the survival time (table III).

Material and Methods

The patients are described in chronological order in table II. In the analysis of the personal history particular notice was taken of any drug, insecticide, glue, dye, hairspray or other chemical substance that might be associated with aplastic anaemia. The nature of the first symptom (anaemia, haemorrhage, or infection) is recorded, as well as the interval between this complaint and the first known blood counts.

Laboratory examination had included routine peripheral blood counts and May Grünewald-Giemsa stained smears. Reticulocyte counts performed after cresyl blue staining were corrected for haematocrit (46% in men, 41% in women), and are recorded as reticulocyte index (18). When possible, similar data had been collected 4–12 weeks after diagnosis.

Vitamin B_{12} and folic acid serum levels had been assayed by standard techniques. Bone marrow cytological preparations obtained by aspiration from various sites had been stained according to standard methods and semiquantitatively evaluated for the presence of haematopoietic cells. Special care was taken to exclude all patients in whom the presence of malignant cells was suspected. Bone marrow biopsy specimens had been obtained with Westerman-Jensen needles or larger bone marrow trephines. After decalcification and paraffin embedding, histological sections were prepared. In 1972, methylacrylate embedding technique described by Burckhardt [7], became available in our hospital. In general, the biopsies were evaluated for loss of haematopoietic tissue and the presence of fibrosis. The results were scored as aplastic or hypoplastic; extensive collagen fibrosis was not found in any of the biopsies. The methylacrylate technique permitted more detailed description of infiltrating non-haematopoietic cells, which is discussed elsewhere [44].

Routine chemical analysis had included kidney and liver function tests and serum protein electrophoresis performed initially on paper and later on cellulose acetate. The concentration of γ-globulin, expressed as grams/litre, is shown in table II.

Additional investigations performed in most of the patients included HbF estimations and cytogenetic studies in the bone marrow of the indicated patients. Tests for auto-immune phenomena, e.g. ANF and direct globulin tests had been performed in 36 patients according to standard techniques. The results are given in table VII. Several investigations had not been carried out in all patients, e.g. bone marrow biopsy and cytogenetic studies. In addition to these shortcomings the methods used for several tests had been altered during the 10 years covered by the survey.

For all of the parameters used, the statistical difference between groups I and II was calculated. Because the prognosis of patients in group III was considered to be

Table II Relevant data for the present series of 40 patients suffering from aplastic anaemia, admitted bet 1964 and 1975 survival from 1st examination the observation period ended 1 1 1976

Patient No.	Age years	Sex	D_x year	Aetiology[1]	Present symptom[2]	Interval weeks[3]	Initial counts Hb g/dl[4]	R	WBC/ µl	PMN/ µl	Plat s/ µl	After 1 3 months Hb g/dl[4]	R	WBC/ µl	PMN/ µl	P µ
Group I survival < 6 months																
1	43	m	1965	idiopat	H, A	4	9 7	8	2,500	600	4 000	10.3	0	2,500	200	10
2	38	f	1966	idiopat	H	1	9 0	3	450	200	5,000	8.0	1	390	100	0
3	59	m	1966	gold	H	2	2.6	6	3 000	600	1 000	4,2	3	1 520	400	9
4	70	m	1966	CAP	H	1	5 4	2	1 800	500	8 000	5 8	1	500	100	9,
5	7?	m	1966	CAP	H	1	12.9	0	1,200	500	5,500	9 0	0	1 100	100	32,
6	30	m	1967	CAP	H/I	1	11 7	0	1 000	10	9 000	8.5	4	4,000	360	17
7	70	m	1969	CAP buta	A	8	6.0	17	5 500	2,800	10,000	7 7	40	3,300	1,300	15,
8	57	m	1972	gold	H	1	8 6	0	2,500	900	10,000	5 6	0	780	16	1
9	60	f	1973	idiopat	H	6	8.9	5	1 700	700	6,600	5.3	0	2,600	200	1
10	21	f	1974	hepatitis?	H	8	8.7	27	3 900	1,500	5 000	5 0	14	3,100	1,500	20,
11	13	f	1975	hepatitis	H	1	12.3	0	3,000	500	57 000	8.1	0	260	20	1
Group II survival > 6 months																
12	32	f	1964	CAP	H	2	6.4	14	1 800	1 100	26,000	9.2	5	1 500	400	15 0
13	26	m	1966	idiopat	A	8	7 7	23	3,900	1,500	7 500	10.2	4	3 000	1 000	10,0
14	56	m	1966	idiopat	A	8	5 4	5	1 400	500	150,000	7 1	2	1,200	480	20,0
15	66	m	1966	CAP	H	4	7 8	3	2,500	50	4 000	6.8	3	3,000	600	6,
16	13	f	1967	idiopat	A	8	7 1	-5	1,940	940	20,000	6.4	20	1,950	720	20,
17	44	f	1968	idiopat	A	8	8.1	5	1 800	1 000	260,000	10.2	18	5 700	1,500	200,
18	50	f	1970	buta	H	6	6.9	–	1 700	900	800	4.8	20	1 630	700	5,00
19	50	m	1970	benzene?	H	1	10.8	20	3 000	4,000	22,000	9 7	15	3,200	500	17 00
20	62	f	1970	idiopat	H	3	8.0	2	2,200	1 100	18,000	16.0	8	3 400	800	2,5
21	14	m	1971	idiopat	A	8	7 0	2	1,300	170	3 700	9 5	23	2,800	140	40,000

Table II (continued)

BM cytol.	BM histol.	Cytogen.	HbF[9]	AID	Gamma-glob. g/l	Therapy + duration[10]	Survival months	Cause of death[11]
0				DCT 1/64	10.3	Sx (2) *andr 3 months*	4	I
0	–			circ. A-C	18.0	predn DCT 1/64	2	I
1		–		neg.	11.3	andr 3 months predn, 3 months	3	H
1				neg.	8.1	predn	4	I
0	*1*	–		neg.	–	*predn, 10 days*	*1*	*I*
0	–	–		neg.		–	3	H
1	1	–	6.4	ANF dub. DCT dub.	17.3	andr 2 months	2	H
1	0			neg.	9.2	predn, 1 month	1	H + renal failure
1	0	breaks		ANF dub.	13.8	Sx (1) andr 2 months	3	H
1	0		10.6	neg.	9.8	predn, 4 months andr 4 months	4	I
1	0			neg.	6.9	predn	1	H, I
0				neg	9.3	Sx	+ 123	
1	0			neg.	9.0	Sx (6)	34	H
1				neg.	22.1	predn, 48 months	49	I?
1			1.3	neg	13.2	predn, 12 months andr 48 months	+ 119	
0			19.0	ANF dub.	7.6	predn, 36 months	+ 104	
1		+1C	1.5	neg.	5.9	predn, 36 months andr 12 months	60	haemo-chromatosis
1	1		2	ANF dub.	7.2	andr 54 months	+ 61	
0			3.4	ANF dub.	9.1	Sx (1) andr 1 month	+ 63	
1	1	47/45	3.8	neg.	9.3	andr 33 months	+ 63	
1	0		9.2	neg.	10.2	andr 45 months	+ 54	

Table II (continued)

Patient No	Age years	Sex	D year	Aetiology[1]	Present symptom[2]	Interval weeks[3]	Initial counts Hb g/dl[4]	R	WBC/µl	PMN/µl	Plat/µl	After 1–3 months Hb g/dl[4]	R	WBC/µl	PMN/µl	Pl µl
22	14	m	1971	idiopat	A H	8	7 7	30	3,200	1 100	20,000	8.0	40	2,400	800	
23	24	f	1971	idiopat	H	2	8 4	54	5 000	3 000	22,500	5.8	30	3,300	1,500	
4	29	f	1971	idiopat	A	8	5.1	8	2,100	1 000	56,000	7.5	4	2,100	700	
25	68	m	1971	buta	A	4	4 8	5	8 100	1 000	135 000	4 9	28	5,400	1,300	
26	22	f	1972	SLE? carbam	A H	3	[illegible]9	4	1,500	280	2,500	6.6	20	4 100	640	1
27	61	m	1973	idiopat	A	12	8.7	4	1,260	270	0	9 5	18	900	180	10,
28	64	m	1973	idiopat	A, H	8	8.8	19	1 700	570	5,000	10.4	60	1 630	930	2,
29	47	m	1975	idiopat	A	4	4 0	10	2,000	380	9 000	8.0	7	[illegible],400	1,200	8
Group III BMT																
30	37	f	1967	idiopat	H/I	2	6.9	3	1 600	800	12,000	6.8	14	940	500	13,
31	23	f	1969	CAP	H	1	9 9	1	1 100	40	20,000	7 4	0	790	0	12,
32	16	m	1970	idiopat	H	8	5 7	42	2,600	800	15,000	7 3	20	1 600	600	30,-
33	26	f	1970	cotrim?	H	8	4 0	3	1,200	500	1 500	7 0	0	4,500	130	7
34	18	m	1971	idiopat	H	1	6.9	2	700	94	4,000	7 6	3	2,800	300	1,5
35	47	f	1972	gold	H	1	8.9	2	3,500	1 000	3,000	8.9	0	1 000	0	
36	14	f	1974	idiopat	H	2	9.5	50	3 100	2,400	1 000	7 9	2	370	270	22
37	19	f	1974	idiopat	H	1	8 6	1	600	0	[illegible],500	8 6	0	1 230	30	2,-
38	23	m	1974	idiopat	A	4	6.8	2	1,500	300	8 000	6.3	30	2,120	520	1
39	50	f	1974	buta	H		11.8	1	2,700	1 130	2,500	5 8	0	1 060	40	7
40	42	f	1975	idiopat	H	4	8.1	72	3,200	1,800	10,000	6.5	0	760	80	10

Table II (continued)

BM cytol.	BM histol.	Cytogen.	HbF[9]	AID	Gammaglob. g/l	Therapy + duration	Survival months	Cause of death[11]
1		n	8.0	neg.	10.8	Pyrid, 10 months	+ 55	
1		+ C, + D	5.0	neg.	12.5	andr 16 months	21	H + I
1	0		8.0	neg.	5.6	andr 9 months Sx (5)	20	H
1			1	neg.	12.2	andr 7 months	12	hepatitis cardiac failure
0	0	XXX	2.5	ANF LE V DCT	12.9	predn, 3 months ara 36 months	+ 40	
0	0	47/	1	neg.	12.3	andr, 23 months	+ 26	–
1	I		3.0	neg.	12.8	andr 17 months	18	I + renal failure
0	0		0.9	neg.	8.3	andr 6 months pyrid, 26 months p-5-P 2 months	+ 10	
1				neg	9.0	BMT (twin)	5	I
0				ANF dub.	10 0	BMT (1)	1.5	I
1	1	breaks	9.2	neg.	12 6	pyrid, 1 month andr 44 months BMT (60)	60	H
0				neg.	9.2	andr 2 months BMT (2)	2	H
0	0		1	neg.	7 3	andr 6 months BMT (7)		hepatitis
0	0		0.7	ANF (+)	15.5	andr 1 month BMT (1)	1	H, I
I	1		1 4	neg	9 7	andr 13 months BMT (1,5)	+ 16	
0	0		7	neg.	10.5	BMT (2) (3,5)	7	GvH
1	0	+ 6	4 3	neg	9.6	andr 6 months BMT (6), Sx (7)	+ 12	
0	0		0.5	neg	8.2	andr 5 months BMT (2)	+ 5	I
1	0			neg.	7 1	andr 1 month BMT (2) Sx (3)	+ 9	

very bad, the difference between I and III together and group II was also calculated. The statistical analysis was made by a corrected χ^2 test at one degree of freedom. The levels of significance are mentioned at the appropriate places.

Results

Figure 1 shows the actuarial survival curve of groups I and II together as well as that of the whole patient group The mean ages of the patients in these groups did not differ statistically although in group III (BMT patients) the average age was lower than in the other two groups (table III)

As shown in table Va and b haemorrhage (H) was more common as a presenting symptom in group I although not significantly The interval between the onset of the haemorrhage and the first blood count tended to be shorter in patients belonging to group I In several patients the haemoglobin and platelet levels had been raised by transfusions before the complete blood count was recorded This means that in these cases the reticulocyte index and neutrophil count are the most reliable indications for the effective haematopoietic function As shown in figure 2, the correlation

Table II (continued)

[1] Aetiology CAP = chloramphenicol buta = butazolidine cotrim = cotrimoxasol idiopat = idi carbam = carbamazepine SLE = systemic lupus erythematosus.

[2] Presenting symptom A = anaemia weakness, fatigue, etc. H = haemorrhage including nose haematuria and petechiae I = infection fever chills, positive blood cultures.

[3] Interval between presenting symptom and first complete evaluation of the peripheral blood.

[4] R = Reticulocyte index = $\dfrac{\text{Retic. count } ^0/_{00} \times \text{Ht}}{46\ (m)\quad 41\ (f)}$

[5] BM cytology = cellularity graded 0–4.

[6] BM histology studied in bone marrow biopts by various techniques, cellularity grade 0–4 – = done.

[7] BM cytogenetics – = not done + C, D etc. = chromosomal abnormalities (for details, see table V n = normal.

[8] HbF percent of Hb.

[9] AID = Auto-immune disease laboratory tests suggestive of auto-immune diseases direct antigamma globulin test (DCT), anti-nuclear factor (ANF), LE phenomenon circulating anti-coagulant (circ. AC).

[10] Therapy andr = androgens (oxymethalone, methalone, metandrostenolone, testosterone proprionate, nortestosterone) predn = prednisone (various dosages) pyrid = pyridoxine 500 mg dd P 5-P = pyridoxal-5-phosphate Sx = splenectomy BMT = bone marrow transplant (attempt) (the interval Dx BMT or Dx-Sx is indicated between parentheses in months) aza = azathioprine.

[11] Cause of death H = haemorrhage, I = infection GvH = graft-versus-host disease.

Table III. Classification of 40 patients, according to survival

Group	Survival	Patients, n			Age ± SD
		total	male	female	
I	< 6 months	11	7	4	48.2 ± 21.2
II	> 6 months	18	10	8	40.5 ± 18
III	BMT[1]	11	3	8	28.3 ± 13.5

[1] Patients prepared for or treated with BMT

Table IV. Prognosis according to period of diagnosis

Period of diagnosis	Groups		
	I	II	III
1964–1969	7	6	2
1970–1975	4	12	9

The prognosis of group III was estimated to be the same as that of group I. There is no significant difference in prognosis between groups I + III vs. II.

between low initial counts and a poor prognosis is not significant. However the same parameters determined 4–12 weeks later in the same patients showed a significant difference between groups I and II ($p < 0.05$).

Cases of drug-associated aplastic anaemia were significantly more numerous in group I, as can be seen from table Vc ($p = 0.04$). In 3 of the 4 cases associated with Butazolidin® or one of its derivates, the patient died. 4 of the 6 patients previously treated with chloramphenicol [CAP] died. The prognosis showed no correlation with the total dose of this drug. All patients with gold- (3) or hepatitis- (2) associated aplasia died within 6 months. 1 patient (No. 26) showing severe pancytopenia and bone marrow atrophy had a positive direct antiglobin test as well as a positive antinuclear factor and LE phenomenon in the absence of any visceral or cutaneous manifestation of SLE. This patient had also taken carbamazepine over a period of several months for post-traumatic epilepsy. A complete remission was obtained after cessation of the carbamazepine and the institution of immunosuppressive treatment, all serological tests have become negative.

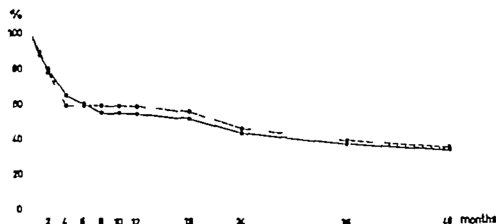


Fig 1 Survival aplastic anaemia.

A complete loss of haemopoietic parenchyme, as determined in the initial bone marrow smears or biopsy specimens, did not show correlation with prognosis.

The HbF level was determined in 23 patients again no correlation with prognosis was found

Chromosomal abnormalities were demonstrated in 7 out of 21 cases (table VIa) No constant pattern could be found (table VIb) No difference was found between groups I and II but complete restoration was not obtained in any case showing chromosomal aberrations. A striking feature is that up to the time of writing no evidence of acute myelogenous leukaemia is found Positive direct antiglobin tests were found in 3 patients. One of these, as already mentioned, also showed strongly positive antinuclear factor and LE tests without evidence of visceral or cutaneous SLE. A circulating anticoagulant (antithromboplastin) had been demonstrated in another patient (table VII) The significance of a weakly positive direct Coombs test and the presence of ANF in the other patients is not clear The serum γ-globulin concentration was recorded as a rough indication of the humoral immunological capacity This concentration had been determined in 38 cases. The mean values was 10 45 g/l ± 3 45 No significant difference was found between groups I II and III

In group I the most common causes of death were haemorrhage and septicaemia. In the patients of group II who died additional causes of death were renal cardiac, or hepatic failure Two of the patients in whom bone marrow grafting was attempted died of thrombocytopenia during

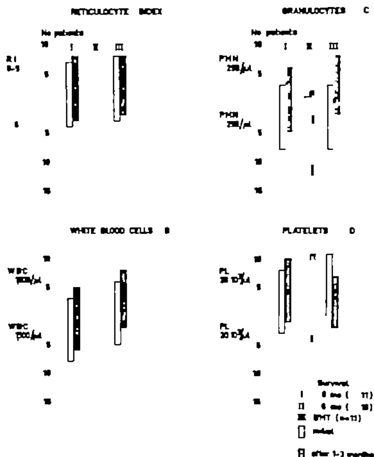


Fig 2 Prognostic parameters in aplastic anaemia.

ALG treatment before transplantation (No. 32 and 33). One succumbed to graft-versus-host disease (No. 37). Again haemorrhage was the most common cause of death in this group. A more detailed description of the various forms of treatment is given elsewhere [15]

The prognosis of the patients who were alive 6 months after diagnosis is shown in table VIII. The patients whose granulocyte count remain under 1,000/mm³ and platelet count under 20,000/mm³ and who require transfusions obviously have a bad prognosis. The causes of death in these

Table Va. Prognosis of aplastic anaemia in relation to presenting symptoms

Presenting symptom	Group I (n = 11)	II (n = 18)	III (n = 11)
Anaemia	1	7	2
Haemorrh. (H)	10	11	9
Infection + Haemorrh.	2	–	–

Table Vb Prognosis in relation to the interval between first sign (H) and first blood counts (Dx)

Interval (H Dx)	Group I	II	III
< 1 week	6	3	6
> 1 week	4	8	3

H = Haemorrhage.

Table Vc. Prognosis in relation to aetiology in aplastic anaemia

Drugs	Group I (n = 11)	II (n = 18)	III (n = 11)
CAP	3	2	1
Buta + derv	1	2	1
Gold	2		1
Cotrimoxasol[1]			1
Benzene[2]		1	
Misc. analg.[3]			1
Hepatitis	2		
Auto-immune[4]	1	1[5]	
Idiopathic	2	12	6

Groups I II and III see table III CAP = Chloramphenicol buta. + deriv = phenylbutazone derivatives.

[1] A relationship between this drug and the aplasia in this case has not been established with certainty

[2] A relationship between this substance and the aplasia was not established.

[3] Including phenylbutazone and many other analgetics.

[4] Definite evidence of auto-immune phenomena

[5] This patient had also received carbamazepine.

Table VIa. Prognostic significance of chromosomal aberration, studied in 21 patients

Chromosome analysis	No abnorm.	Abnormalities
Failure / Partial remission	9	7
Complete remission	5	0

6–30 metaphases evaluated.
Abnormalities detailed in table VIb.
Failure: persistent pancytopenia partial remission Hb level elevated to > 10 g% neutrophils > 1,000/μl but platelet count remaining below 50,000/μl.
One case of XXX included.

Table VIb. Nature of the chromosome abnormalities found in and out of 21 aplastic anaemia patients

Patient No.	
9	breaks, gaps
17	+ C
20	hyperdiploid and hypodiploid metaphases, unspecified
24	+ C + D
28	+ C
33	breaks, gaps, and deletions
38	+ C (No. 6)

patients are related to the disease (septicaemia and haemorrhage) as well as to the treatment (hepatitis and haemochromatosis).

Discussion

The definition of aplastic anaemia given above was reached by the exclusion of known primary disorders. It is clear that many different pathogenetic mechanisms must play a role in the development of the haematopoietic failure of aplastic anaemia. A rapidly progressive, acute type and a gradual chronic type can be distinguished on the basis of the survival curves of several series [20, 28, 29 32] including the present one

Table VII Occurrence of signs of 'auto-immunity' in the present aplastic anaemia series

	I	II	III
Direct antiglob. test			
Negative	5	16	11
Weak	1	1	
Positive	2	1	
ANF			
Negative	5	14	10
Weak	2	3	1
Positive		1[1]	0
Circ. anti-coag.	1		

[1] Also LE-positive.

Table VIII Prognosis of aplastic anaemia in 18 patients surviving 6 months from diagnosis

	n	†[1]
Persistent pancytopenia + Transfusion requirement[2]	7	6
Recovery of blood counts	11	1

[1] Survival = median 27 months (range 12–61 months) cause of death septicaemia 3 cases haemorrhage 1 case hepatitis 3 cases.
[2] Transfusion substitution of red cells and platelets.

Any retrospective clinical survey covering the last 10 years will be affected by changes in additive therapy e.g. tissue antigen matching for blood component therapy and the modifications applied to antibiotic treatment. We could find no significant difference between the first and second 5 year periods in this series. A more detailed account of the treatment of aplastic anaemia will be given elsewhere [15]

As a basis for the selection of the proper treatment for patients who will die in an early stage of the disease, we tried to single out prognostic parameters in our group of patients retrospectively The decision to separate the patients into two groups, one with a survival of 6 months or less and the other with a survival of more than 6 months was based upon the

course of the survival curve. LYNCH *et al.* [29] took 4 months of survival as criterion on the basis of the survival in their series. In contrast to others [24 32] we found that a poorer prognosis was observed in drug-associated cases. The statistical significance was borderline, possibly due to the small numbers. In fact, the designation 'idiopathic' is only an admission of our ignorance [17] The association of drugs with aplastic anaemia can be divided into three categories. certain, probable, and not probable [47] We included only drugs that have been reported in the 'certain' and 'probable' categories. In our series all patients with gold-associated aplasia showed a rapidly fatal course which is in agreement with the findings of WOHLENBERG [48]

Chloramphenicol was associated with aplastic anaemia in 6 patients, 4 of whom died very soon, thus underlining the risk involved in the use of this drug. WALLERSTEIN *et al* [45] estimated the risk of the development of aplastic anaemia after CAP therapy to be 12–13 times greater than that of the general population. The pathogenetic mechanism is unclear YUNIS [49] showed that CAP inhibits mitochondrial protein synthesis in physiologic concentrations. SUMM *et al.* [41] found no significant difference between this inhibitory action of CAP and that of tetracycline. To explain the different myelotoxicities of these drugs they suggested the existence of a special condition for CAP-influx into the mitochondria of bone marrow cells. Our data do not allow conclusions as to the incidence of CAP-induced aplastic anaemia, but they are in agreement with the prognosis indicated by BEST [6]

Phenylbutazone and its main conversion product oxyphenbutazone have been reported in association with agranulocytosis, thrombocytopenia, and aplastic anaemia [14 30] The prognosis of these cases did not differ from the idiopathic pattern. In our series the prognosis seems to be more serious, probably due to the small number of cases. In 1 patient, phenylbutazone was among the many analgetic drugs taken to alleviate headaches. 1 patient had been given cotrimoxazole for 2 months prior to the onset of severe aplastic anaemia, and showed progression despite adequate supepletion of folinic acid. In this case, the association of the drug with the aplastic anaemia is equivocal.

Viral hepatitis is also associated with very severe aplastic anaemia. ALTOUNI and DOBBLIN [1] reviewed 88 cases and found a mortality of more than 88% 10 months after diagnosis. The hepatitis-aplastic anaemia syndrome shows the age and sex patterns of viral hepatitis, i.e., it occurs mainly in male patients below the age of 30. In most cases the progressive

pancytopenia develops within 9 weeks after the clinical onset of the hepatitis [1] Summarizing these findings, it appears that in most patients, the knowledge of aetiological factors was not helpful in estimating the prognosis in individual cases, although in our experience drug associated cases tend to have a worse prognosis than idiopathic aplasias. The magnitude of the insult to the early progenitor cells is reflected in the degree and duration of the pancytopenia, and could be prognostic importance. Although data concerning *in vitro* evaluation of haematopoietic progenitor cells have been reported, no clear-cut relationship with the prognosis has been demonstrated [11 22] Haemorrhage as presenting sign and especially within a week before diagnosis tends to occur more often in patients with a bad prognosis [29 32] This may be correlated with a drastic decrease of the peripheral counts and thus with a sudden onset of the disease. From our findings as shown in figure 2, it is clear that a bad prognosis is correlated with the persistence of severe pancytopenia for more than 4–12 weeks, as reflected in the granulocyte count and reticulocyte index. As also observed by others [20] neither cellularity of the bone marrow smears nor the presence of haemopoietic parenchyme in biopts showed correlation with the prognosis. In a limited number of patients the presence and extent of infiltration by lymphocytes, plasma cells, mast cells, and histiocytes proved to parallel the severity of the disease [44] The evaluation of combined histological and clinical data as prognostic index is under current investigation. It appeared to be impossible to evaluate bone marrow smears reproducibly with respect to the relative presence of non myeloid cells in our material as described by LI *et al* [25] and LYNCH *et al* [29]

Several authors have speculated about a role of autodestructive immunity in the pathogenesis of aplastic anaemia [3 12, 19 33 39 43] Despite extensive studies, no evidence for antibody antigen complexes has been found in the bone marrow of patients suffering from aplastic anaemia [36] A cellular cytotoxic mechanism was suggested in 1 patient described by ASCENSAO *et al* [2] We encountered a few patients who fulfilled all of the criteria for aplastic anaemia and also exhibited auto-immune haemolysis. We found no conclusive evidence for an increased incidence of auto-immune phenomena, e.g. on the basis of direct anti-globulin test and the presence of anti nuclear factor

2 patients showed clear-cut indications of auto-immunity (No 2 and 26). In patient No. 26 carbamazepine was also implicated as an aetiological factor This drug has been known to be associated with aplastic anae

mia since 1964 [38] and its role in the pathogenesis of the severe aplastic anaemia in this patient is not proven. After discontinuation of the drug and under prolonged immune-suppressive therapy the anti-nuclear factor and direct anti-globulin titre gradually disappeared in parallel with a complete recovering of haematopoiesis. It should be noted that carbamazepine has also been reported in association with a relapse of systemic lupus erythematosus [26]

A few authors have stated that an immunological incompetence is part of the aplastic anaemia syndrome [31 37] The only relevant parameter that could be evaluated in most of the patients in our series was the serum concentration of total gammaglobulin. In contrast to MORLEY and FORBES [31] we did not find a significant hypogammaglobulinaemia.

Chromosomal abnormalities in aplastic anaemia are often regarded as a pre leukaemic sign. Although it is generally assumed that aplastic anaemia may terminate in acute leukaemia [9 27] this evolution was not seen in the large series reported by NAJEAN *et al.* [32] KEISER [20] and WILLIAMS *et al* [46] possibly due to the problems involved in the differential diagnosis of bone marrow insufficiency syndromes, especially with respect to aleukaemic leukaemia, as discussed by BERNARD [4] BEARD *et al.* [5] and LEWIS [24] Alternatively it might be an expression of the heterogeneity of this rare disease.

In a still incomplete prospective study PIERRE [34] has found no significantly increased incidence of acute leukaemia in patients with aplastic bone marrow and cytogenetic abnormalities. It was striking, however that none of the patients in our series with complete recovery of the blood counts, showed cytogenetic changes. This might be of importance with respect to the pathogenesis of aplastic anaemia. Hypothetically a chromosomal abnormality in the bone marrow cells could indicate a defect in the haematopoietic stem cell. The origin is unknown, but in our cases the defect seems to be acquired. The abnormalities themselves appeared to be stable during prolonged periods. Hypothetically some drugs could induce certain chromosomal abnormalities similar to those found in rabbits treated with benzene [21] and in man after butazolidine treatment [40] HASHIMOTO *et al* [16] found evidence of a disturbed DNA repair mechanism in *the lymphocytes of aplastic patients, which might indicate that these individuals* are more susceptible to drug-induced chromosome abnormalities.

So far no consistent pattern of chromosomal aberration has been distinguished as typical for aplastic anaemia. Chromatid or chromosomal breaks and gaps may be related to a deranged repair mechanism, whereas

pancytopenia develops within 9 weeks after the clinical onset of the hepatitis [1]. Summarizing these findings, it appears that in most patients the knowledge of aetiological factors was not helpful in estimating the prognosis in individual cases, although in our experience drug-associated cases tend to have a worse prognosis than idiopathic aplasias. The magnitude of the insult to the early progenitor cells is reflected in the degree and duration of the pancytopenia, and could be prognostic importance. Although data concerning *in vitro* evaluation of haematopoietic progenitor cells have been reported, no clear-cut relationship with the prognosis has been demonstrated [11, 22]. Haemorrhage as presenting sign and especially within a week before diagnosis tends to occur more often in patients with a bad prognosis [29, 32]. This may be correlated with a drastic decrease of the peripheral counts and thus with a sudden onset of the disease. From our findings as shown in figure 2, it is clear that a bad prognosis is correlated with the persistence of severe pancytopenia for more than 4–12 weeks, as reflected in the granulocyte count and reticulocyte index. As also observed by others [20], neither cellularity of the bone marrow smears nor the presence of haemopoietic parenchyme in biopts showed correlation with the prognosis. In a limited number of patients the presence and extent of infiltration by lymphocytes, plasma cells, mast cells, and histiocytes proved to parallel the severity of the disease [44]. The evaluation of combined histological and clinical data as prognostic index is under current investigation. It appeared to be impossible to evaluate bone marrow smears reproducibly with respect to the relative presence of non-myeloid cells in our material, as described by LI *et al.* [25] and LYNCH *et al.* [29].

Several authors have speculated about a role of autodestructive immunity in the pathogenesis of aplastic anaemia [3, 12, 19, 33, 39, 43]. Despite extensive studies, no evidence for antibody antigen complexes has been found in the bone marrow of patients suffering from aplastic anaemia [36]. A cellular cytotoxic mechanism was suggested in 1 patient described by ASCENSAO *et al.* [2]. We encountered a few patients who fulfilled all of the criteria for aplastic anaemia and also exhibited auto-immune haemolysis. We found no conclusive evidence for an increased incidence of auto-immune phenomena, e.g. on the basis of direct anti-globulin test and the presence of anti nuclear factor.

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aneuploidy results from non-disjunction. This is in agreement with the aetiologic heterogeneity mentioned above The unchanged pattern and extent of the abnormalities we observed may indicate the switching on of a pathologic clone of stem-cell able to survive for prolonged periods. This may be correlated with the finding of persistent abnormalities in peripheral blood counts, bone marrow histology and colony forming capacity *in vitro* [17 19 44] The possibility that potential leukaemic disorders are hidden in this group cannot be ruled out [35] To exclude paroxysmal-nocturnal haemoglobinuria sucrose-lysis and acid lysis tests were performed repeatedly *These tests invariably gave negative results.*

We conclude from the results of the present retrospective study that aplastic anaemia probably comprises a heterogeneous group of diseases manifested by haematopoietic insufficiency The prognosis within the first year after diagnosis seems to be related to the degree and duration of the pancytopenia in the early period after diagnosis. At present, no other parameter for the extent of the damage to the bone marrow and the possibilities as regards its repair are available Spontaneous complete recovery is rare and many long term survivors show persistent abnormalities. The presence of cytogenetic aberrations in the bone marrow preparations seems to be correlated with incomplete recovery Conventional tests for auto-immunity give negative results in the majority of the patients. We found no evidence of hypogammaglobulinaemia in our patients.

To select the proper treatment, further investigations are needed to clarify the pathogenetic mechanisms and to determine the prognosis.

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aneuploidy results from non-disjunction This is in agreement with the aetiologic heterogeneity mentioned above. The unchanged pattern and extent of the abnormalities we observed may indicate the switching on of a pathologic clone of stem-cell able to survive for prolonged periods. This may be correlated with the finding of persistent abnormalities in peripheral blood counts, bone marrow histology and colony-forming capacity *in vitro* [17 19 44] The possibility that potential leukaemic disorders are hidden in this group cannot be ruled out [35] To exclude paroxysmal-nocturnal *haemoglobinuria, sucrose lysis and acid lysis tests were performed* repeatedly These tests invariably gave negative results.

We conclude from the results of the present retrospective study that aplastic anaemia probably comprises a heterogeneous group of diseases manifested by haematopoietic insufficiency The prognosis within the first year after diagnosis seems to be related to the degree and duration of the pancytopenia in the early period after diagnosis. At present, no other parameter for the extent of the damage to the bone marrow and the possibilities as regards its repair are available. Spontaneous complete recovery is rare, and many long term survivors show persistent abnormalities. The presence of cytogenetic aberrations in the bone marrow preparations seems to be correlated with incomplete recovery Conventional tests for auto-immunity give negative results in the majority of the patients. We found no evidence of hypogammaglobulinaemia in our patients.

To select the proper treatment, further investigations are needed to clarify the pathogenetic mechanisms and to determine the prognosis

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Congenital Hypoplastic Anaemia with Unusual Dyserythropoietic Features

A Case Report

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Key Words. Hypoplastic anaemia Congenital dyserythropoiesis Blackfan-Diamond's syndrome Bone marrow hypoplasia Erythroid dysplasia

Abstract A case of severe congenital anaemia (Hb levels 3–4 g/dl) is presented. The patient, a 21 year-old female, requires, since the age of two, periodical blood transfusions, but is nevertheless able to lead a normal life as a housewife. Although the case has to be considered as a form of congenital hypoplastic disorder of the erythroid tissue some of the clinical and haematological features are compatible with a congenital dyserythropoietic state. On the basis of clinical findings it is suggested that it may represent a previously undescribed form of anaemia which shares the features of both hypoplastic and dysplastic congenital affections of erythropoiesis.

The term congenital hypoplastic anaemia is used to define a group of rare haematological disorders. The patients show a severe anaemia with a marked erythroid hypoplasia of the bone marrow These disorders have at least in some instances, a familial occurrence and usually manifest in early childhood [2] They may undergo spontaneous or therapeutic remissions [1 3 6] Although bone marrow hypoplasia is the dominant feature, dysplasia (i e qualitative defects of erythropoiesis) is present at a variable extent [8]

In congenital dyserythropoietic anaemia the dysplasia is, however the main feature. In these cases the erythropoiesis is markedly hyperplastic but inefficient [10]

The author suggests that intermediate conditions exist between congenital hypoplastic and dyserythropoietic anaemias. He presents a case of

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congenital anaemia in which hypoplasia of the erythroid tissue is accompanied by striking dyserythropoietic features.

Case Presentation

The patient, Vincenza N., a 21-year-old girl, was first referred to the Haematology Department of Reggio Calabria Hospital for a routine transfusion in January 1975. Her history had apparently started at the age of 2–3 years, when she first presented with anaemia. From that period on, the patient received regular transfusions (approximately 300 blood transfusions before she came to our care). Her disease was not investigated until 1972, when the girl sought medical assistance in a University Hospital in Northern Italy. In the course of this hospitalization the diagnosis of 'severe anaemia with hypoplasia of the bone marrow' was made and a course of androgens and corticosteroids was undertaken, however, without any detectable benefit.

Physical examination upon admission to our department did not reveal any abnormal finding except marked pallor of skin and mucosae. The patient is a very attractive and intelligent girl, with regular menses since the age of 15. She has never complained of breathlessness, fatigue, or laziness during her usual housewife activities, in spite of severe anaemia. The patient lives with her parents in a small village at 600 m altitude, and, when in need of a blood transfusion, she travels alone to the hospital by coach.

Laboratory Investigations

On her first admission in 1975, the laboratory investigations yielded the following results: Hb 3.2 g/dl, RBC 760,000/mm³, MCV 105 µm³, Ht 13 %, WBC 6,500/mm³, platelets 200,000/mm³, reticulocytes 0.6 %, serum iron 280 µg/dl. The blood film revealed marked anisocytosis with macrocytosis. No siderocytes. Osmotic fragility normal, no abnormal haemoglobin at the electrophoretic pattern, no tetramers. Hb A_2 3.8 %, Hb F 7.4 %.

Serology. Blood group 0 (CDe/CDe), Kell negative, Cellano, Duffy, M, N, P positive, S negative. Coombs' test negative. Acidified serum lysis negative with ten donor sera and patient's own serum. Cold antibody lysis negative with ten control sera. Anti-I agglutination negative.

Blood volume (^{51}Cr). Red-cell volume: 445 ml (11 ml/kg); plasma volume: 2,680 ml (63 ml/kg), total blood volume 3,125 ml (74 ml/kg).

Ferrokinetics (^{59}Fe). T ½ clearance: 140 and 139 min at an 8-months interval (normal 60–140 min); plasma iron turnover: 1.34 mg/100 ml blood/day (normal 0.4–0.8); red cell iron turnover: 1.33 mg/100 ml blood/day (normal 0.3–0.8); red cell utilization: 40.6 % by day 17; surface counting: increased liver uptake, scanty bone marrow and spleen uptake, suggestive of secondary siderosis (fig. 1, 2).

Trephine biopsy. Haemopoietic components markedly reduced, increased adipose tissue.

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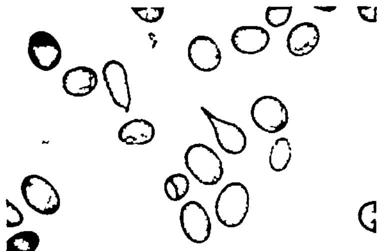


Fig. 3. Peripheral blood film.

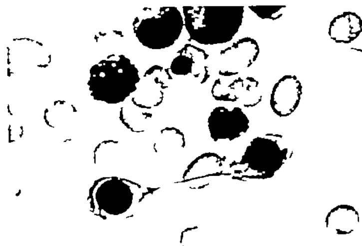


Fig. 4 Bone marrow: cytoplasmic strands between erythroblasts.

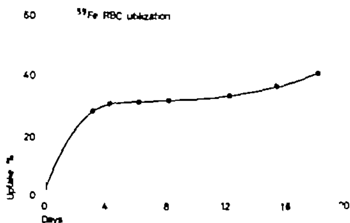


Fig 1 Red cell incorporation of 59Fe: 40.6% by day 17

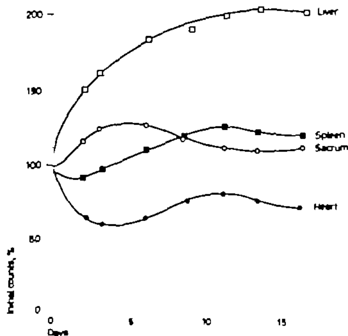


Fig 2 Surface counting after a single intravenous injection of 59Fe. The radioactivity is expressed relative to the radioactivity measured in the same organ 30 min after the introduction of the isotope

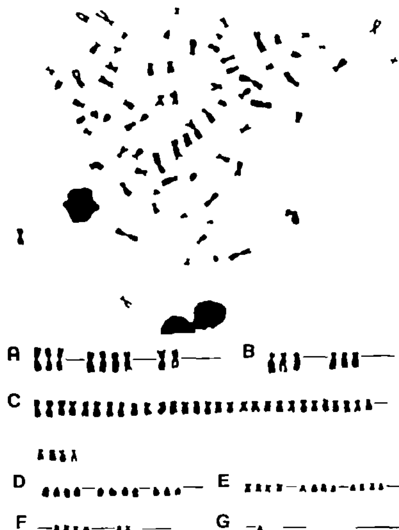


Fig 6 Hyperdiploid bone marrow metaphase.

amine is administered at regular intervals because of suspected post-transfusional siderosis (the patient refused a liver biopsy). Androgens, corticosteroids and pyridoxine have proved completely ineffective. When at home, in spite of the severe anaemia, the patient is able to lead a normal life.

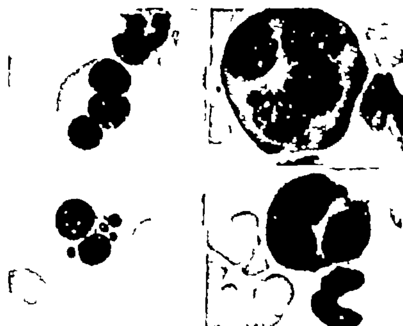


Fig 5 Bone marrow different aspects of erythroblastic multinuclearity

Bone marrow smears Normal cellularity granulocytic/erythroblastic ratio slightly superior to normal. Megakariocytic series normal, high incidence of mast cells. The erythroblastic series shows marked dyserythropoietic alterations, such as multinuclearity cytoplasmic bridges between erythroblasts, kariorrhexis, and pyknosis (fig. 3–5).

Ch omosome studies Normal chromosome patterns were obtained from cultures of peripheral lymphocytes stimulated with phytohaemagglutinin (PHA) Bone marrow cultures showed, out of 36 metaphases examinated, 21 normal and 15 aneuploid mitoses (10 with 80–92 chromosomes, 3 with 43–44 chromosomes, and 2 with 49 (fig. 6)

Electron microscopic studies Binucleated erythroblasts with abnormal nuclear shape were found. Anomalies of the nuclear cisterna and the sponginess of chromatine are not prominent Figure 7 shows a binucleated erythroblast with margination of chromatin the nucleolus is adjacent to the perinuclear membrane that lso shows irregular disruptions. (The E. M studies were done with the help of Dr G TONIETTI, II Clinica Medica, Rome's U iversity Hospital.)

Family investigations Haematological investigations on the p tient parents were normal two apparently normal brothers are living abroad

Follow-up The patient has been hospitalized for 4- to 5-week periods in October 1975 and February 1976 in order to carry out more detailed and time-consuming haematological investigations. She otherwise needs packed red cell transfusions every 5–7 weeks, when her Hb levels f ll under 4 g/dl. After nearly 2 years follow-up, no significant haematological modifications have been observed (fig 8) Desferriox

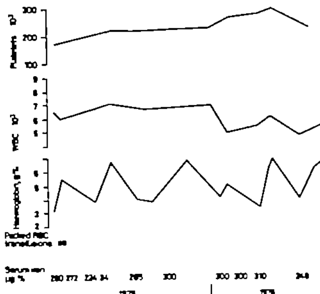


Fig. 8. Haematological follow-up of the patient from January 1975 to September 1976. Folic acid and B_{12} vitamin were unable to modify Hb levels.

Discussion

Analysis of the anamnaestic, haematological and clinical findings suggests, at first, the diagnosis of congenital hypoplastic anaemia.

Fanconi's type of anaemia is ruled out on account of the normal appearance of the megakaryocytic and myeloid series in the marrow, the absence of somatic abnormalities of the limbs or of the genitourinary tract [7], the normal chromosome pattern obtained with PHA-stimulated peripheral lymphocytes [5], and the negative family history [4, 9].

It is likewise easy to discard a thalassaemic or thalassaemia-like disorder, as well as a congenital form of sideroblastic anaemia (a hereditary sex-linked disorder).

A diagnosis of Blackfan-Diamond's syndrome is suggested by the early onset of the disorder, the low haemoglobin levels without leucopenia or thrombocytopenia, the constantly increased levels of Hb F, and the increased number of mast cells in the marrow with normal myeloid and megakaryocytic series.

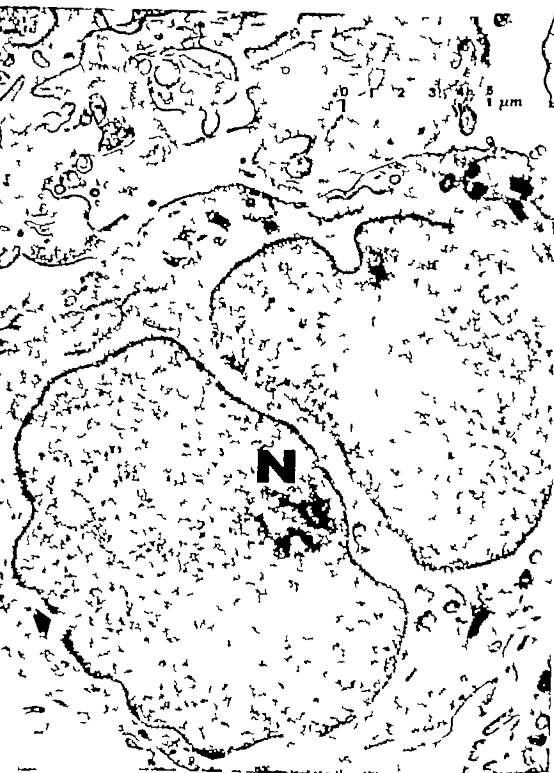


Fig 7 Electron microscopy: binucleated erythroblast × 22,000

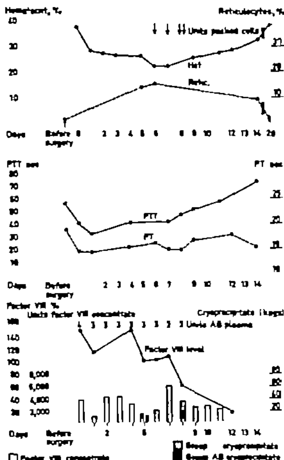


Fig. 1. The clinical course after herniorrhaphy. PT = Prothrombin time; PTT = partial prothrombin time; Hct. = hematocrit, Retic. = reticulocyte count.

Case Report

A 57-year-old black man (blood group AB, Rh-positive) with hemophilia A was admitted to Cook County Hospital on September 18, 1975, for an incarcerated right inguinal hernia. Significant past history included several hospital admissions for hemarthroses, hematuria, and gastrointestinal bleeding, heavy alcohol intake, heroin abuse, and advanced cirrhosis of the liver. Physical examination revealed gynecomastia, spider nevi, hepatomegaly (4 cm), an incarcerated right inguinal hernia, and

The diagnosis of Blackfan Diamond's syndrome is, however unsatisfactory erythroid tissue in the marrow aspirate is conspicuous, dyserythropoiesis is marked and remained a constant feature during the 2 years follow-up The long duration of the affection the relative benignity and the lack of response to steroids and androgens are in contradiction with this diagnosis.

The dyserythropoiesis is shown by morphological signs as binuclearity and multinuclearity cytoplasmic strands between erythroblasts, karyorrhexis, pyknosis, irregular fragmentation of the perinuclear membrane in the erythroblasts. The ineffective erythropoiesis, demonstrated by the ^{59}Fe investigations also suggests the diagnosis of congenital dyserythropoietic anaemia The absence of erythroblastic hyperplasia and hyperbilirubinaemia, however make the diagnosis of congenital dyserythropoietic anaemia difficult while the serology (normal acid lysis and normal agglutinability with anti i and anti-I) is not compatible with the classical forms of the disease

We suggest that the morphological appearance of the marrow the haematological results, and the kinetic data of our patient are due to a previously undescribed form of anaemia that shares some of the clinical and haematological features of both hypoplastic and dysplastic congenital affections of erythropoiesis.

Another point of interest is supplied by the chromosome alterations encountered in the bone marrow These could, in fact, suggest an evolution into a myeloproliferative disorder although nearly 20 years duration of the disease and the repeated bone marrow examinations make it difficult to believe in an evolution to malignancy

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olysis of the recipient's cells does occur, infusion of group 0 cells would avoid hemolysis of the donor cells. Fortunately, such hemolytic reactions have not caused any serious problems [1, 2, 5]. Presently, the risk of such hemolytic reactions is a justifiable price to pay for ensuring hemostasis in the hemophiliacs undergoing surgical procedures. However, efforts must be made to increase the supplies of group-specific cryoprecipitate. Alternatively, methods of manufacture of commercial factor VIII should be modified to eliminate plasma units with high anti-A and anti-B antibody titers from the plasma pool. Even the possibility of making group-specific factor VIII concentrates should be explored.

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a reducible left inguinal hernia. Hemoglobin was 11.6 g%, hematocrit 37%, reticulocyte count 2.1%, prothrombin time 19 sec, partial thromboplastin time 57 sec, fibrinogen 220 mg%, factor VIII level 5%, no factor VIII inhibitor serum albumin 2.7 g%, globulin 5.4 g%, total bilirubin 2.9 mg% (direct bilirubin 1.8 mg%). The patient received 3,900 U of commercial factor VIII and 4 U of group AB fresh frozen plasma preoperatively with demonstrable normalization of the coagulation profile. Bilateral herniorrhaphy was performed. Subsequent blood component therapy coagulation parameters, hematocrit and reticulocyte counts are depicted in figure 1

On the first postoperative day the hematocrit dropped to 28% and steadily declined to 22% on the sixth day without any bleeding. The patient developed febrile reaction after infusions of factor VIII and fresh frozen plasma. On the fifth post operative day jaundice (total bilirubin 6.8 mg%, 2.6 mg% direct), sperocytes, positive Coombs test (direct and indirect), anti-A antibody (1 4 titer), no anti B antibody hemoglobinemia and hemoglobinuria were detected. The commercial factor VIII which was obtained from a single lot, contained anti-A and anti B antibodies in 1 128 and 1·64 titers, respectively With four units of packed red cells, he maintained the hematocrit at 26%. Replacement therapy with group AB cryoprecipitate, group A cryoprecipitate and factor VIII concentrate was given for 11 days. Subsequently fever subsided, jaundice cleared and, 2 weeks later hemoglobin was 11.8 g%, reticulocyte count 3%, total bilirubin 1 7 mg% (direct 0.8 mg%).

Comment

Surgical procedures can be carried out in hemophiliacs without excessive bleeding if proper replacement therapy is given. In our case, the bleeding diathesis on account of the cirrhosis of the liver also had to be corrected simultaneously

Hemolysis occurred after the infusion of commercial factor VIII and persisted as long as he received the concentrate Anti A antibody was found to be the cause of hemolysis. All the fresh frozen plasma was group-specific, so it is not likely to be the cause of hemolysis.

When large amounts of factor VIII are needed to treat hemophilia patients of blood group A, B or AB it would be preferable to use type-specific cryoprecipitate, as it would eliminate the risk of such hemolytic reactions. One of the drawbacks in using this product is that individual units of cryoprecipitate vary widely in factor VIII content. Hence, factor VIII assays must be intermittently performed to monitor the infusion therapy For surgical procedures requiring large amounts of factor VIII limited supplies of group-specific cyroprecipitates may pose an additonal problem. At present, one has to depend on the commercial factor VIII concentrates from pooled plasma to achieve hemostasis in such cases. If hem

olysis of the recipient's cells does occur infusion of group 0 cells would avoid hemolysis of the donor cells. Fortunately such hemolytic reactions have not caused any serious problems [1 2, 5] Presently the risk of such hemolytic reactions is a justifiable price to pay for ensuring hemostasis in the hemophiliacs undergoing surgical procedures. However efforts must be made to increase the supplies of group-specific cryoprecipitate. Alternatively methods of manufacture of commercial factor VIII should be modified to eliminate plasma units with high anti-A and anti-B antibody titers from the plasma pool. Even the possibility of making group-specific factor VIII concentrates should be explored.

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Further Observations on the Incidence and Properties of Lymphocytotoxins in Leukaemia[1]

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Key Words Lymphocytotoxin Leukaemia

Abstract 54 out of 112 patients with leukaemia have been shown to produce a lymphocytotoxin in their serum when the number of malignant cells present in the peripheral blood was considerably raised but the normal blood cells were low This lymphocytotoxin reacts with all normal cells. It is a high molecular weight protein, though not an antibody Partial purification was achieved by elution from DEAE 52-cellulose at about pH 6.9

It has been previously reported [1] that a cytotoxic substance was present in the serum of 15 out of 51 patients with leukaemia. It was detected only before or early during, cytotoxic treatment or during a relapse and was called a lymphocytotoxin (LCT) due to its activity upon detection. In this paper the findings on its incidence are confirmed by the testing of a further 61 patients, bringing the total investigated to 112, and more recent work on its characterization and action is discussed.

Methods

Screening of Sera

The cytotoxicity test of TERASAKI and MCLELLAND [2] was modified by omitting the addition of complement for use in the screening of sera for the presence of LCTs. The end point of this test is the lysis of the lymphocytes which is shown by the uptake of a blue stain, when an LCT is present. Sera from each of the 112 patients were tested in this way on each visit to the hospital, each being tested against lymphocytes from at least 20 normal donors. Control sera from 33 normal individuals and 90 patients with various other diseases were similarly tested.

Absorption Experiments

No lysis of granulocytes or erythrocytes by LCT-positive sera was detectable so tests were carried out to show whether absorption with these cells removed LCT ac-

1 Supported by a grant from Leukaemia Research Fund.

tivity from the sera. To do so, pure preparations of lymphocytes, granulocytes and erythrocytes were made from normal blood by the technique of BØYUM [3], after which the granulocytes and erythrocytes were separated by sedimentation. For each absorption 1 part of packed cells was mixed with 2 parts of LCT-positive serum and incubated for 2 h at 37 °C; the supernatant was then recovered by centrifugation, and tested against lymphocytes in parallel with the same unabsorbed sera.

In Vitro *Effect on Living Cells*

The effect of the LCT on lymphocytes in culture was looked at since this was nearer the *in vivo* situation than the lymphocytotoxicity test. A modification of the method of MOORHEAD *et al.* [4] for PHA stimulated lymphocyte cultures was used substituting RPMI for TC 199 I all cases the cell cultured were group O Rhesus positive. In each test a comparison was made between (1) cells + PHA + AB+ve serum (normal control) (2) cells + PHA + leukaemic serum, (3) cells + AB+ve serum (no PHA), and (4) cells + leukaemic serum (no PHA).

All cultures were set up in triplicate, incubated for 72 h, labelled for ½ h with ^{125}I 5-iodo-2'-deoxyuridine solution (IDU), and then harvested [5].

Immunoglobulin Inhibitions

In the previous paper [1], a method for inhibition by anti-human IgG or anti-human IgM was described. Subsequently this was extended to include anti-human IgA, anti-human IgD and broad spectrum anti-human immunoglobulin. LCT-positive sera were incubated for 1 h at 37 °C with one of the anti-human immunoglobulin sera before testing against normal lymphocytes. The amount of anti-human immunoglobulin added was *in excess of the amount required to inhibit strong HLA antisera* by the same method. All the anti-human immunoglobulins were tested in parallel against the same LCT sera and the same cells except the anti-IgD which had to be tested later

Molecular Size

The molecular size was determined by passing LCT-positive sera through series of membranes on the Amicon Diaflo system, under pressure of 8 lb/in² In each experiment the volume was reduced first to half then to quarter of the original. Both the concentrate and filtrate were then tested for LCT activity

Purification of Sera

(a) As result of the molecular weight experiments G-200 column [6] 37.5 × 2.5 cm was used with 0.01 M phosphate buffer at pH 8.0 5 ml NaH_2PO_4 (1.56 g/l) + 95 ml $Na_2HPO_4 \cdot 2H_2O$ (1.779 g/l) – as eluting fluid for purifying the LCT A 2-ml sample, *dialysed overnight against the phosphate buffer*, was run on the column, with small aliquots of blue dextran and paranitrophenol as markers of known molecular size. All columns were run at 4 °C and 15-min fractions (a volume of about 8 ml) were collected.

(b) A DEAE 52-cellulose column [7] with pH gradient obtained by continuous addition of 0.01 M $NaH_2PO_4 \cdot 2H_2O$ to starting solution of 0.01 M phosphate buffer at pH 8.0 (as above) was an alternative method of purification. Once gain the col-

umn was run at 4 °C and 15-min fractions were collected, but in this case the fractions were pooled in to 13 groups of 10 tubes each. The pH of each pool was measured and the pH adjusted to 7.0 by the addition of 0.01 M Na_2HPO_4 and concentrated to 2 ml before testing

(c) Absorption and elution of LCT serum using both lymphocytes and platelets according to the technique of Shulman *et al.* [8] was also used in the attempts to purify the LCT A range of 0.1 N HCl/0.1 N NaOH buffers from pH 4.0 to 12.0 was used to find the optimum pH for the eluting fluid.

Results

Incidence and Reactivity of the Lymphocytotoxin

The existence of a cytotoxic substance in the serum of certain patients with leukaemia was reported previously [1] and the study of a further 61 patients has confirmed those results As table I shows, a total of 112 patients have now been tested of which 54 showed positive lymphocytotoxic reactions. In the original study none of the 7 patients with acute lymphoblastic leukaemia (ALL) produced an LCT but recently the serum of one ALL patient showed strong LCT activity in the few weeks prior to his death. Another recent ALL patient showed weak LCT activity but this patient, an adult, was not a typical ALL and the results obtained were inconclusive The results, therefore, show that the LCT can occur with all types of leukaemia but may be less common in ALL. Among the controls no normal individuals (33 tested) showed LCT activity Of 90 patients, with a variety of diseases including other forms of cancer other haematological diseases and unrelated diseases such as glandular fever measles and rubella tested 5 showed LCT activity Of these 5 patients 1 had lymphosarcoma, 1 had polycythaemia with a busulphan induced aplasia, and 1 was the IgE-deficient neutropenic boy discussed by Ng and Prankerd [9] The remaining 2 patients were also neutropenic but were not available for further study The LCT has also been detected in a patient in a pre-leukaemic state and in 2 patients with myeloid metaplasia who subsequently developed acute myeloid leukaemia (AML)

When the presence of LCT was correlated with the stage of the disease, it was always found to be associated with an excessive number of abnormal cells in the peripheral blood. Usually the number of normal cells was also depressed though on occasions this effect was masked by the artificial raising of the cell counts by transfusion. Figure 1 shows the blood counts of a patient with chronic myeloid leukaemia (CML) throughout the course of his disease, and is typical of the picture found.

Table I Distribution of lymphocytotoxin among different types of leukaemia

Type of leukaemia	Total number tested	Number LCT + ve
Acute myeloid	45	23
Chronic myeloid	16	6
Acute lymphoblastic	11	2
Chronic lymphatic	38	23
Stem cell	1	
Acute (undifferentiated)	1	–
Total	112	54

Table II Reaction by absorption of lymphocytotoxin and normal blood cells

	Testing cells from 6 donors, %					
	1	2	3	4	5	6
Serum RM						
Neat serum	44	35	36	21	50	34
Adsorbed with						
Lymphocytes	<5	<5	<5	<5	<5	<5
Granulocytes	<10	<5	<5	<5	<10	<5
Erythrocytes	34	<5	14	<5	<10	<5
Serum GW						
Neat serum	45	50	44	36	33	50
Adsorbed with						
Lymphocytes	<5	<5	<5	<10	<5	<5
Granulocytes	<10	<5	<5	<10	<10	<5
Erythrocytes	<10	<5	<10	<10	<5	<5

The association of the LCT in the serum with a depressed number of normal cells as well as a raised blast cell count suggested that it reacted with normal cells. The results of the absorption tests are shown in table II and from these it seems clear that the LCT reacted with granulocytes and erythrocytes as well as lymphocytes. Earlier evidence also suggested a reactivity with platelets [1] though under the conditions of the lymphocytotoxicity test it would not kill leukaemic blast cells.

When the effects of the leukaemic sera on normal lymphocytes in culture under PHA stimulation were investigated considerable variation was found (table III). However this variation could not be correlated with the

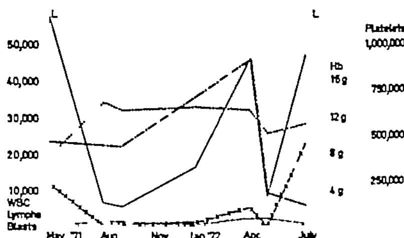


Fig 1 Patient R. M. with chronic myeloid leukaemia. At the onset of the disease, before treatment, the white cell count (—) was 57,000/cm³ of which 12,000/cm³ were blast cells. The lymphocyte count (- - -) was 500/cm³ platelets (—) were 470,000/cm³ and the haemoglobin ( ) was 7 g/100 ml, and an LCT was detected (L). Treatment greatly reduced the number of blast cells and caused fluctuations in the normal cell number The LCT disappeared and was only detectable again at the end of the course of the disease when the blast count (×—×) had risen to 24,000. At that time the platelet and lymphocyte counts were very low (85,000 and 500/cm respectively). Haemoglobin was 11.2 g/100 ml but the patient had been transfused. O = Tested with negative results.

presence or absence of the LCT and is more probably attributable to a substance such as the colony stimulating factor described by Hadden [10]

Properties of the Lymphocytotoxin

It was suggested [1] that since the LCT was destroyed by trypsin it was a protein, though other factors discussed in the earlier paper indicated that it was not an antibody The experiments with the anti-human immunoglobulin (table IV) showed that neither the broad spectrum anti-human immunoglobulin nor any of the more specific anti-immunoglobulin sera inhibited LCT activity showing that it was not an immunoglobulin, and thus confirming that it was not an antibody

To obtain further information on the nature and properties of the LCT it was desirable to isolate it from the other serum proteins. The first step in this procedure was the estimation of its molecular size, and using the Amicon Diaflo system it could be shown that this was > 100 000 (table V) This fact further pointed to a protein substance and suggested that Sephadex G-200 chromatography might be a suitable method for purifi

Table III. Correlation between LCT occurrence and affect on PHA response of leukaemic serum

Patient	LCT status	PHA response
CLL	+	inhibited
AML	−	inhibited
ALL	−	inhibited
AML	+ʷ	unchanged
AML	−	augmented
AML	−	augmented
ALL	+	inhibited
AML	+	augmented
AML	+	unchanged
AML (1)	−	augmented
(2)	+	augmented
AML	+	unchanged
AML	+ʷ	augmented
AML	+	augmented
CLL	−	unchanged

+ = Strongly positive +ʷ = weakly positive + = positive − = negative.

cation. Of the markers used, blue dextran, with a molecular weight of 2 million, came off immediately and all the subsequent fractions were collected, concentrated to 2 ml, and tested, but no activity was detected. After several attempts, all with a negative result, it was concluded that the LCT must be absorbed onto the Sephadex G-200. This was confirmed by mixing equal volumes of LCT-positive serum and Sephadex G-200 in a beaker for 1 h, centrifuging and retesting the supernatant. The result showed that the LCT activity had been absorbed by the Sephadex, a result that was surprising since Sephadex is generally considered to be an inert substance.

On ion exchange chromatography on DEAE 52-cellulose, using the pH gradient described above for elution, LCT activity was detected in the 10th and 11th pooled fractions (table VI). The pH of fraction 10 was 6.965 and that of fraction 11 was 6.85 immediately on elution from the column, considerably lower than the pH of 8.0 required for the elution of antibodies.

The absorption and elution experiments using lymphocytes and platelets brought surprisingly negative results despite the wide range of pH used. This suggested that the LCTs reaction with a cell was irreversible.

Table IV Anti-human immunoglobulin inhibition tests (percent cells killed)

Serum (LCT + ve)	Control	Serum reacted with broad spectrum anti-immunoglobulin	Serum reacted with anti-IgG	Serum reacted with anti-IgM	Serum reacted with anti-IgA	Control	Serum reacted with anti-IgD
GB (1)	25	21	48	32	30	33	33
TB (1)	21	24	24	19	18	28	32
AC	26	19	21	19	15	–	–
IG	58	65	60	62	61	75	80
JM	37	42	57	50	56	40	41
RM	23	23	23	21	18	50	50
JO	33	33	32	36	32	50	54
GW (1)	31	35	46	35	31	50	50
T Wint	30	26	37	30	30	–	–
TW	<10	<10	<10	<10	<10	50	50
Control antisera							
Anti HLA A1	+	–	–	–	–	+	–
Anti-HLA A2	+	–	–	–	–	+	–
Anti HLA A8	+	–	–	–	–	+	–

Discussion

The results of these experiments have shown that the LCT is not an antibody but that it is a large molecule, probably a protein, with a molecular weight of > 100 000 The LCT can be purified by DEAE 52 ion exchange chromatography when the activity was recovered as a single peak at about 6 9

This study has confirmed the earlier results [1] that the LCT was present in the serum of patients with AML, CML and CLL (chronic lymphatic leukaemia) and in addition 2 patients with ALL have shown LCT activity Numerous controls were studied no normal individuals showed LCT activity and it could be detected only in a few non leukaemic patients. 3 of these patients with an LCT had other haematological malignancies, while the final diagnosis of 2 was not known These results indicate that most commonly the presence of an LCT is associated with leukaemia.

Table V Treated against 10 donor's lymphocytes

Original serum	PM 10 concentrate	PM 10 filtrate	PM 30 concentrate	PM 30 filtrate	XM 100 concentrate	XM 100 filtrate
+ + + +	+ + +	/	+ + +	+	+ + +	/
+ + +	+ + +		+ + + +	(+)	+ + +	
+ + +	+ + +	+	+ + + +	+	+ +	
+ + +	+ + +	/	+ + +	(+)	+ + +	/
+ + +	+ + +	/	+ + +	/	+ + + +	
+ + +	+ + +	/	+ + + +	/	+ + +	
+ + + +	+ + +	/	+ + + +	(+)	+ + +	/
+ + +	+ + + +	/	+ + +	+	+ + +	/
+ + +	+ + +	/	+ + +		+ + +	/
+ + + +	+ + +	/	+ + + +	/	+ + + +	(+)

+ + + + + = 95% + + + = 85–95% + + + = 75–85% + + = 60–75% + + = 40–60% + = 20–40% + = 10–20% (+) = 5–10% / = 5% PM 10 cut off point = MW 10,000 PM 30 cut off point = MW 30,000 XM 100 cut off point = MW 100,000.

Table VI Reactivity present in fractions from DEAE 52 column tested against 10 donors lymphocytes

Original serum	Fraction No. and pH 1	2	3	4	5	6	7	8	9	10	11	12	13
	8.0	7.89	7.77	7.66	7.54	7.43	7.31	7.20	7.08	6.96	6.85	6.74	6.5
+	/	/	/							/	(+)	/	+
+ + +	/	/	/	/	(+)				/	+ +	/	+	/
+ + +	/	/						/		+ +	/	/	+
+ + +	/	/	(+)	/	/		/	+	/	+	(+)	/	
+ + +	/	/	/	/				/		+ +	+ +	/	/
+ + +	/	/	/				/	(+)	/	+	/	/	
+ +	/	/	(+)	/			/	/		(+)	+		
+ +	/	/		/						+	(+)	/	
+ +	(+)	/		/			/	/	(+)	(+)	+ +	/	
+ + +	/	/		/		(+)	/		(+)	+	+ +	+	/

For interpretation of key see table V

The presence of an LCT appears to coincide with an increase in the number of malignant cells in the peripheral blood and a corresponding decrease in the number of normal cells The latter is not always as clear cut as the former especially after the commencement of treatment. This may be due to the suppressive effects of cytochemical drugs on all cells or to the raising of the cell numbers, especially erythrocytes, after transfusion

The finding that the LCT is a specific large molecular weight protein associated with a high leukaemic cell count suggests that it is made by these cells It is absorbed by normal blood cells, and in the case of lymphocytes at least it lyses them but does not react with leukaemic cells. This suggests that the LCT may be partly responsible for the low white (normal) cell counts, thrombocytopenia and anaemia found in leukaemia. It could be a substance of biological significance in enhancing the spread of the disease and its control may improve the prognosis of the patient.

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Macroglobulinemia of Waldenström Associated with Severe Osteolytic Lesions

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Key Words. Macroglobulinemia Osteolytic lesions

Abstract. Osteolytic lesions are not know to be associated with chronic lymphocytic leukemia and are rare in macroglobulinemia of Waldenstrom I the present paper we report patient suffering from macroglobulinemia associated with chronic lymphocytic leukemia in whom osteolytic lesions, resembling those of multiple myeloma were found. Treatment with chlorambucil resulted in normalization of the peripheral blood smear but the malignant infiltration of the bone marrow continued and manifested itself by appearance of new osteolytic lesions, though some of the old lesions underwent recalcification after treatment.

Macroglobulinemia of Waldenström, first described in 1944 [11] was considered to be free of osteolytic lesions and this criterion was used to differentiate it from multiple myeloma [2, 3 10 12] Recently several reports [4 6 7 9 10] have indicated that osteolytic lesions are part of this disease spectrum. In the present paper we report an unusual case of macroglobulinemia associated with chronic lymphocytic leukemia in whom severe osteolytic lesions resembling those of multiple myeloma were found. These lesions underwent partial recalcification after treatment.

Case Report

M. R 68-year-old woman was hospitalized elsewhere in 1969 for generalized lymphadenopathy splenomegaly and 20,000 WBC/μl with 80° lymphocytes (table

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Table I Laboratory findings

Date	HB g/dl	WBC per µl	Mono %	Lymph %	Band %	Neutr %	Eosin	IgG mg/dl	IgA mg/dl	IgM mg/dl
1969		20 000	1	80	1	16	2	–		
Oct. 1973	13	13 000	2	63	2	29	4	–		
Dec. 1973	9 5	3,500	?	45	?	?	?	470	76	380
June 1974	13 4	9 200	4	22	?	67	5	–	–	
March 1975	11 6	4,200	17	5	–	76	–	620	109	190

l) A diagnosis of chronic lymphatic leukemia was made and no treatment was given.

In 1972 she was admitted for severe oral moniliasis. Her bone marrow was infiltrated with small lymphocytes and a monoclonal IgM was detected in the serum. She was given chlorambucil and remained asymptomatic until 1973 when she was admitted to Hadassah University Hospital because of bone pain and extreme fatigue. The main physical findings were pallor palpable masses on the skull, enlarged lymph nodes in the neck, axillae and groins and palpable liver (3 cm) and spleen (5 cm) below the costal margin

Her sedimentation rate was 85/120, hemoglobin 9.5 g/dl, WBC 3,500/µl with 45% lymphocytes (table I) Total protein 6.1 g/dl with albumin 3.2 g/dl and globulin 2.9 g/dl Calcium 7.5–8 mg/dl and alkaline phosphatase 550 IU/ml, mainly of bone origin (normal 80)

On serum electrophoresis there was a monoclonal protein fraction between the β and the γ-region, which was identified as IgM type K (fig. 1) The serum level of IgG was 470 mg/dl IgA 76 mg/dl and IgM 380 mg/dl (table I)

A skeletal survey revealed osteolytic lesions in the skull (fig. 2), distal part of right humerus and radius and compressed fracture of the 12th thoracic vertebra The bone marrow was infiltrated by small lymphocytes and plasma cells.

The patient was diagnosed as suffering from Waldenström's macroglobulinemia and was treated with chlorambucil, vitamin D and calcium supplement. During the following 6 months, the Hb level rose to 13 4 g/dl WBC 9,200 with a normal differential count (table I) The tumor masses in the skull disappeared and the osteolytic lesions declined in size (fig. 3) The patient discontinued treatment on her own and 2 months later a tumor mass appeared in the right mandible which on biopsy

Fig 1 Immunoelectrophoresis of the patient serum in the central well and of a normal control serum in the outer well The lower trough contains an a t whole human serum and the upper trough an anti-IgM serum Note the pathological configuration of the IgM of the patient marked by an arrow

Fig 2 X ray of the skull before treatment Note the extensive osteolytic lesions.

Fig 3 X-ray of the skull after treatment Note the recalcification of many of the lesions.



1



2



3

proved to be a non-Hodgkin lymphoma. It regressed following local X-ray irradiation.

The patient remained well on chlorambucil therapy until November 1974 when she complained of severe low back pain and difficulty in walking. X-ray examination disclosed osteolytic lesions in the right ilium Local irradiation resulted in a mild symptomatic relief

In January 1975, she was readmitted because of recurrence of tumors in the skull Hepatosplenomegaly was evident, without lymphadenopathy The paraprotein persisted. The immunoglobulin levels were IgG 620 mg/dl, IgA 109 mg/dl and IgM 190 mg/dl Calcium 12.2 mg/dl and alkaline phosphatase 170 IU/ml

The patient was given combined chemotherapy with BCNU endoxan, leukeran and prednisone. In addition she was treated parenterally with fluids, corticosteroids and furosemide. However she died in hypercalcemic coma within several days.

Discussion

Macroglobulinemia was first described by WALDENSTRÖM in 1944 [11] Since then several reviews dealing with this disease have been published [2–4 6–10 12] The association of macroglobulinemia with chronic lymphocytic leukemia has been reported [13] and it has been suggested that macroglobulinemia and chronic lymphatic leukemia are the expressions of the same disease entity The finding of IgM markers on the surface of th lymphocytes of chronic lymphatic leukemia lend further support to this contention.

Our case started as chronic lymphatic leukemia with an IgM paraprotein. Treatment with chlorambucil arrested or rather cured the leukemic process but did not arrest the proliferation of the cells in the bone marrow which led to osteolytic lesions in different bones. Osteolytic lesions in macroglobulinemia are considered to be very rare [2, 3 8, 10 12] However recently several reports have been published stressing the presence of small osteolytic lesions in this disease [4 6 7 9] Our case is conspicuous in that the osteolytic lesions were very large and are compatible with those found in multiple myeloma. This diagnosis was ruled out by repeated bone marrow biopsies, which showed the presence of lymphoid cells characteristic of a lymphomatous process.

Therapy with chlorambucil or X rays, together with vitamin D and calcium led, in the beginning to the disappearance of the tumors of the skull and the mandible and recalcification of the osteolytic lesions Thus is most remarkable and to the best of our knowledge it has not been reported previously

LEE *et al* [5] have treated 2 patients with macroglobulinemia of Waldenström with a combination therapy of BCNU cytoxan, alkeran, prednisone and vincristine with good results. Similar results were obtained by him and AZAM and DELAMORE [1] in treating patients with myelomatosis. Our experience with this kind of therapy in this case and in another case of macroglobulinemia of Waldenström was disappointing.

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Effect of Cancer Chemotherapy Drugs on Platelet Aggregation in Children

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Key Words Cancer chemotherapy drugs Platelet aggregation

Abstract The effect of vincristine (VCR), cytoxan and actinomycin-D on platelet aggregation was studied in 30 children with solid tumors. h after administration of these drugs, a statistically insignificant reduction in the primary wave of aggregation was observed with the exception of ADP induced aggregation after VCR injection. Unobtainable secondary wave aggregation was seen in 28, 33 and 40% of the patients after administration of VCR, cytoxan and actinomycin-D respectively However none of the patients had clinical bleeding. It was concluded that after the intravenous administration of therapeutic dosages of these drugs, the bleeding tendency caused by inhibition of aggregation is not to be expected.

Cytotoxic drugs are used extensively in the treatment of malignant disease and some have been shown to have an inhibitory effect on platelet aggregation *in vitro* [5 6 10] Although an *in vitro* effect of some cancer chemotherapy drugs shows an *in vivo* correlation with animal studies [5] this has not been clearly shown for human beings.

In this paper we would like to show the influence of vincristine (VCR) cytoxan and actinomycin-D on platelet aggregation function in patients who had solid tumors

Materials and Method

In this study 30 children (20 males, aged -15 years; 10 females, aged 5-9 years) with a variety of solid tumors who had received chemotherapy previously were studied. However none of the patients had had any medications, including cytotoxic drugs, aspirin or others known to interfere with platelet function, during the month prior to the study and had no evidence of bleeding or infection The type of tumors in the patients studied is shown in table I

Venous blood samples were obtained from patients bef re and h after intravenous injections of cytoto ic drugs. The intra venous dosage of VCR, cytoxan and a

tinomycin-D used in this study was, 0.05, 25 mg/kg and 5 µg/kg, respectively. Blood was mixed (9:1) with 3.8% sodium citrat and centrifuged at room temperature for 20 min at 600 rpm to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifuging the remaining blood at 15,000 rpm for 10 min. Platelets were enumerated by phase microscopy using the method of BRECHER and CRONKITE [1]. In all aggregation studies, platelet counts in the PRP samples were adjusted to 350 × 10⁹/1 with autologous PPP.

Platelet aggregation was measured in chrono-log aggregometer and changes in light transmission were recorded continuously on model 702 strip chart recorder by using adenosine-5-diphosphat (ADP- Sigma Chemical Co.) and epinephrine. ADP was dissolved in Tyrode's solution and epinephrine was diluted with saline to obtain final concentration of 1.1 and 2.7×10^{-4} M, respectively.

For statistical analysis aggregation curves were evaluated according t the aggregation angle (A) and percent platelet aggregation (T_{max}) as indicated previously [8]. Aggregation angle gives the initial rate of aggregation, it was determined by measuring the angle between the baseline and the tangent to the initial slope. Per cent platelet aggregation indicates the maximum percent change in light transmission of PRP after the addition of ADP and epinephrine.

Results

The effect of VCR on platelet aggregation was studied in 12 patients, of cytoxan in 10 and of actinomycin-D in 8 patients and the influence on platelet aggregation is shown in figures 1–4.

Study with Epinephrine Induced Aggregation

Before and 2 h after injection of VCR, the mean rates of aggregation angles were, 133.1 ± 11.94 (±1 SD) and 133.7 ± 13.57 for cytoxan 132.0 + 12.52 and 133.5 ± 10.91 and for actinomycin-D 131.5 ± 11.39 and 138.7 ± 12.68 (fig. 1).

Before and 2 h after intravenous injection, the mean percent platelet aggregation (T_{max}) for VCR was 26.2 ± 5.74 (±1 SD) and 25.8 ± 5.37%, for cytoxan 21.2 ± 2.76 and 19.7 ± 2.85%, and for actinomycin-D 24.0 ± 4.56 and 20.5 + 5.30% (fig. 2).

None of the results observed were found to be statistically significant ($p > 0.05$).

Study with ADP-Induced Aggregation

Before and 2 h after intravenous injection the mean rates of aggregation angle for VCR were 118.4 ± 7.75 and 117.7 ± 6.25 for cytoxan 113.4 6.48 and 118.6 ± 9.83 and for actinomycin-D 118.7 ± 6.96 and 120.4 9.54 (fig. 3).

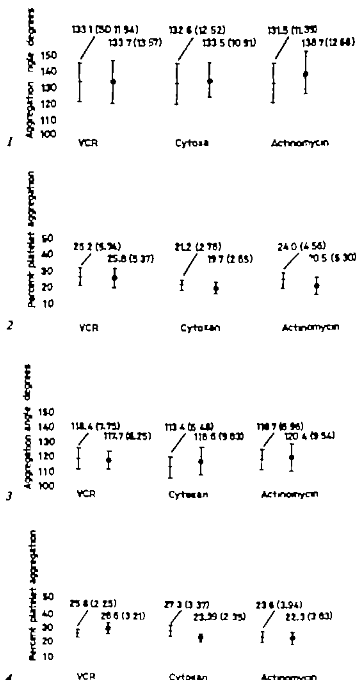


Fig 1 Aggregation angl before and fter the administration of VCR cyto an and actinomycin-D in children with tumors with epinephrine-induced aggregation. O = After chemotherapy

Table I Types of tumors in children studied

Types of tumor	VCR	Cytoxan	Actinomycin-D
Lymphoma	5	6	
Hodgkin's	5	1	
Wilms	1		
Ganglioneuroblastoma	1		
Lymphoepithelioma		2	4
Malignant histiocytosis		1	
Rhabdomyosarcoma			
Osteogenic sarcoma			1
Fibrosarcoma			1

Table II Unobtainable secondary wave platelet aggregation before and after administration of chemotherapy

Drugs	Before chemotherapy	After chemotherapy
VCR	5/12	2/7
Cytoxan	4/10	2/6
Actinomycin-D	3/8	2/5

Number of patients studied.
With ADP and epinephrine

Before and 2 h after intravenous administration, the mean percent platelet aggregation for VCR was 25.8 ± 2.25 and 28.6 ± 3.21 /o, for cytoxan 27 6 ± 3.37 and 23.39 ± .35%, and for actinomycin-D 23.6 ± 3 94 and 22.3 ± 3.83% (fig. 4).

None of the results observed were found to be statistically significant ($p > 0\ 05$).

12 of these 30 children had unobtainable second phase aggregation

Fig 2 Percent plat let aggregation before and after the administration of VCR, cytoxan and actinomycin-D in children with tumors with epinephrine-induced aggregation. O = After chemotherapy

Fig 3 Aggregation angle before and after the administration of VCR, cytoxan and actinomycin-D in children with tumors with ADP-induced aggregation. O = After chemotherapy

Fig 4 Percent pla elet aggregation before and after administration of VCR, cytoxan and actinomycin-D in children with tumors with ADP-induced aggregation O = After chemotherapy

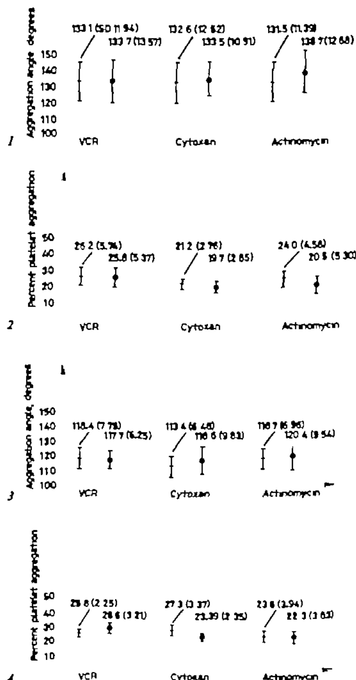


Fig 1 Aggregation gle before od after the administration of VCR cytoxan and actinomycin-D in children with tumors with epinephrine-induced aggregation. O = After chemotherapy

difference has not been explained and probably more studies on this subject are needed.

Our results showed that after intravenous administration of VCR, cytoxan and actinomycin-D some nonsignificant inhibitory effect on primary and secondary wave platelet aggregation could be seen. However none of the patients had clinical bleeding so it was concluded that after intravenous administration of therapeutic dosages of these drugs, the bleeding tendency caused by inhibition of aggregation is not to be expected. Therefore, we would suggest that any bleeding in patients who are receiving these drugs is not caused by a defect in platelet aggregation function.

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prior to administration of drugs In the study with epinephrine and ADP induced aggregation of the 12 patients who received vincristine, 7 showed a second wave aggregation which was suppressed in 2 after the administration of the drug Of the 10 patients who received cytoxan, 6 showed a secondary wave aggregation which was suppressed in 2 after the administration of this drug and from the 8 patients who were given actinomycin D suppression occurred in 2 of the 5 who had secondary wave aggregation (table II)

Discussion

Since in patients with malignant diseases impaired platelet aggregation has been shown [2–4] one would expect that the administration of these drugs might influence their bleeding tendency However most of the previous studies were *in vitro* and none of them clearly showed the clinical importance of impairment of platelet aggregation [5 6] In this study after the intravenous administration of VCR, cytoxan and actinomycin-D some degree of increment in the aggregation angle and some reduction in percent aggregation was noticed but the differences were not statistically significant ($p > 0.05$) With the ADP induced aggregation, except for VCR, some degree of increasing value for the mean aggregation angle and some decreasing effect on percent platelet aggregation was observed but this was also statistically insignificant ($p > 0.05$)

Prior to the administration of the drugs, 40% of our children had unobtainable second phase aggregation As indicated before, by *Steinherz et al* [7] this unobtainable second phase aggregation may have been due to the chemotherapy which had been given previously After the administration of VCR, cytoxan and actinomycin D the absence of secondary wave aggregation was observed in 28 33 and 40% of the patients, respectively with both aggregating agents

Although the mechanism by which these drugs abolishes the second phase aggregation is not clear changes in the surface property of configuration of the cell membrane has been suggested [7 11] The mechanism of the effectiveness of VCR on human platelets has been studied previously [9–11] and *Steinherz et al* [7] indicated that long term administration of VCR in clinical usage has a demonstratable inhibitory effect on platelet function In our study 2 h after the intravenous administration of VCR some decreasing effect with epinephrine-induced aggregation but an increasing effect with ADP induced aggregation was observed This

difference has not been explained and probably more studies on this subject are needed.

Our results showed that after intravenous administration of VCR, cytoxan and actinomycin D some nonsignificant inhibitory effect on primary and secondary wave platelet aggregation could be seen. However none of the patients had clinical bleeding so it was concluded that after intravenous administration of therapeutic dosages of these drugs, the bleeding tendency caused by inhibition of aggregation is not to be expected. Therefore, we would suggest that any bleeding in patients who are receiving these drugs is not caused by a defect in platelet aggregation function.

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A Tentative Classification of Factor XIII Deficiency in Two Groups

To the Editor·

Immunological and immunofluorescent studies were carried out on plasma and platelets of 3 cases of congenital factor XIII deficiency Two of these patients were originally thought to have normal factor XIII subunit S and no subunit A [4] However repeated assays carried out using different lots of antiserum showed that the patients lacked in reality both subunit S and subunit A (fig 1) The false positive result was due to the presence of an anti factor VIII contaminant in the antiserum originally used (Behringwerke lot 2434) That this is so is well demonstrated by the observation that in von Willebrand s disease no second (factor VIII) peak was evident (fig 1)

These two patients seem to be the first cases in whom no factor XIII subunits S is detected The third patient had a normal subunit S and no subunit A In agreement with the above findings are some immunofluorescent studies (fig. 1) No factor XIII antigen was found in fact by the indirect immunofluorescent technique in normal factor XIII deficiency and von Willebrand s disease platelets. On the contrary using the non monospecific antiserum (Behringwerke lot 2434) a fluorescent pattern similar to that observable using an anti-factor VIII antiseum had been noted [1]

On the basis of these observations a tentative classification of factor XIII deficiency in two groups is proposed type I which appears to be rare is characterized by the lack of both factor XIII subunits S and A Type II which appears to be relatively common is characterized by normal and near normal subunit S and no subunit A

The need for a re-evaluation of published cases of factor XIII deficiency by means of monospecific antisera is indicated This is more so if one takes into account the fact that most research work on the subject was carried out using the antisera supplied by Behringwerke Laboratories [2 3 5] The possibility that lot 2434 or similarly contaminated lots of antiserum might have been used remains open

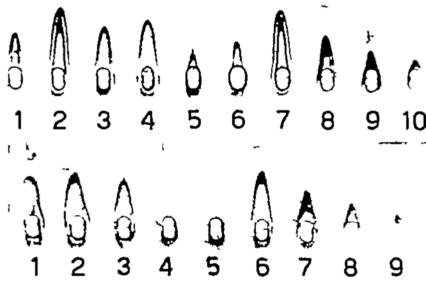


Fig 1 Electroimmunoassay *Top* (contaminated anti-subunit S antiserum): (1) 1:2 diluted pooled normal plasma, (2) undiluted pooled normal plasma (3) von Willebrand' disease; (4) another patient with von Willebrand' disease; (5) factor XIII deficiency (case 1) (6) factor XIII deficiency (case 2, sister of case 1), (7–10) 1:1, 1:2, 1:4 and 1:8 diluted pooled normal plasma. Two major rockets or peaks are evident in normal plasma only one rocket is evident in von Willebrand' disease and in factor XIII deficiency The rockets seen in von Willebrand' disease (wells 3 and 4) are taller as compared to those seen in factor XIII deficiency (wells 5 and 6) The plasma of the 3rd patient with factor XIII deficiency is not reported but showed two rockets as normal plasma. *Bottom* (monospecific anti-subunit S antiserum): (1) von Willebrand' disease (2) another patient with von Willebrand' disease (3) factor XIII deficiency (case 3), (4) factor XIII deficiency (case 1): (5) factor XIII deficiency (case 2, sister of case 1) (6–9) 1:1, 1:2, 1:4 and 1:8 diluted pooled normal plasma. Note that case 3 (well 3) shows a normal subunit S rocket, whereas case 1 and 2 show no precipitate (wells 4 and 5)

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Systemic Mastocytosis a Case Report

Cytological, Cytochemical and Ultrastructural Considerations

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Key Words. Mastocytosis, systemic Mast Cell Splenohepatomegaly

Abstract A case of systemic mastocytosis with unusual clinical manifestations, appearing as an isolated splenohepatomegaly is described. The proliferative character is evident from the cytological characteristics of immaturity and the presence of moderate percentage of mast cells in the peripheral blood. These cells make up 40% of the total cells in the bone marrow

Special attention is given to the optical, morphological, cytochemical, and ultrastructural studies of the disease. Some anomalies were found at the subcellular level which apparently have not been recorded until present.

Various dyshematopoietic features of this case are reported, which may be considered as manifestations of paraneoplastic syndrome

Introduction

Systemic mastocytosis offers a wide pathologic spectrum with diverse clinical manifestations. Urticaria pigmentosa is the most frequently observed condition, and in this case infiltration by mast cells is confined to the dermis. However SAGHER *et al* in 1952 [16] described concomitant bone lesions in urticaria pigmentosa. Mast cells may also invade other organs, especially those that are rich in mononuclear phagocytes, giving rise to what is known as systemic mastocytosis [14 19] Rare cases of this disease may develop without any observable dermatologic lesion [17] When infiltration of the blood and bone marrow by mast cells becomes the predominant finding in systemic mastocytosis, the term mast cell leu

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kemia might be employed [2, 3 7] Solitary mast cells tumors in human beings are exceptional [1]

Aside from urticaria pigmentosa tumor pathology of the mast cell is very infrequent. Therefore, we consider it of interest to publish a clinical case of this disease. Motivation came from two basic circumstances. One was the discovery of certain morphological peculiarities at the ultrastructural level which have been described in a preliminary report [15] though little was found on this subject in the literature. The other reason was a rather exceptional clinical manifestation in the form of an *isolated* splenohepatomegaly which has very seldom been observed [4 8]

Case Report

Our patient was a 44-year-old male without significant personal or pathologic antecedents. He normally consumed 1 liter of wine daily The present illness began 2 years ago with complaints of asthenia, attacks of rhinorrhea, urticarious rash, and sporadic epistaxis. He was admitted to our Department in February 1975.

Physical examination revealed flushing, necrotic lesions in both ears, spontaneous ecchymoses, and a smooth hepatomegaly (7 cm) of firm consistency Likewise, a splenomegaly of 3 cm was noticed. There were no cl nical signs of hepatopathy

The hematological analytical data are shown in table I The predominant findings were pancytopenia, mastocytes in the peripheral blood, and a high percentage of mast cells with immature features in the bone marrow smears. Dyshematopoiesis characterized the blood cytology including a partial sideroachrestic phenomenon (8%/ of ringed sideroblasts) partial degranulation of the polynuclear neutrophils, and a Pelger Huët nuclear anomaly

All of the biochemical determinations were within normal limits, including serum heparin, serotonin, and urinary 5-hydroxyindolacetic acid levels. Skin biopsy revealed a nonspecific chronic vasculitis, but toluidine blue staining failed to show infiltration by mast cells. Thoracic, bone, and gastrointestinal X-rays were normal, too.

The patient s condition worsened with an increase in the splenohepatomegaly and in the bone marrow infiltration by mast cell (t ble I)

The methodology employed to study the proliferation of mast cells included optical and morphological examinations of bone marrow smears and peripheral blood smears with May-Grünwald-Giemsa stain. The following cytochemical tests were performed toluid ne blue, periodic acid Schiff react on (PAS) peroxidase, alkaline phosphatase, β-glucuronidase, naphthol AS-D acetate esterase, chloroacetate esterase, acid phosphatase, and Perl's stain. The conventional tech ique was used to study the ultrastructural pattern the bone marrow aspirate was double fixed in glutaric aldehyde and osmic acid and embedded in Vestopal W

Table I. Hematological results in a case of systemic mastocytosis

Data	Results in February 1975	June, 1976
Erythrocyte count $10^{12}/l$	3.8	3.3
Hemoglobin, g/dl	12.3	9.3
Leukocyte count $10^{9}/l$	3.4	12.2
Differential count		
Neutrophils (filamented), %	33	37
Neutrophils (juvenile), %	19	5
Eosinophils, %	0	0
Basophils, %	0	0
Lymphocytes, %	34	35
Monocytes, %	14	16
Mastocytes, %	0	5
Leukoerythroblastic syndrome	–	+
Platelet count $10^{9}/l$	40	20
Myelogram		
Overall cellularity	+++	++++
Fat	–	–
Erythroblastic series, %	63	40
Granulopoietic series, %	17	20
Megakaryocytic series	++	+++
Mast cell infiltration, %	20	40

Results

A significant infiltration of mast cells in the bone marrow smears was observed (fig. 1). These cells were morphologically different from normal mastocytes. Their size varied from 15 to 25 μm. They were oval, round, or comet-shaped. The granules were found in polar groupings and scarcely covered the nucleus, unlike normal mastocytes. Cytochemical characteristics of these coarse metachromatic cytoplasmic granules are referred to in table II. The nucleus was eccentrically located, showing lax chromatin and the usual presence of nucleoli (fig. 2, 3).

At an ultrastructural level an intense surface activity was seen in the cellular periphery. Cytoplasmic projections may have simulated false cytoplasmic inclusions according to the plane of section (fig. 4, 5). The nucleus had scant heterochromatin and frequent nucleoli. The cytoplasm



1



2



3



Fig 4 Mast cell with multiple cytoplasmic projections or microvilli (arrows). × 6,000. Bar equals 833 nm

Table II Cytochemical analysis of the mastocytes

Stain	Result
Toluidine blue	intense metachromasia
PAS	few cells, weakly positive
Alkaline phosphatase	–
Peroxidase	–
Chloroacetate esterase	very strong positivity
Naphthol AS-D acetate esterase	highly positive
Acid phosphatase	moderate positive (diffuse)
β-Glucuronidase	highly positive (diffuse and granular)
Perl' stain	–

Fig 1 Bone marrow smear. Si mast cells. May-Grünwald-Giemsa. 700.

Fig 2 Peripheral blood smear. Comet-shaped mast cell. May-Grünwald-Giemsa. 700

Fig 3 Bone marrow smear. T mast cells; one of them showing an immature nucleus, the other bearing an erythroblast in its cytoplasm. × 700


1
2
3



Fig 7 Mast cell. The nucleus (N) between two sideroachrestic reticulocytes (R) in the cytoplasm × 5,000. Bar equals 1,000 nm.

contained an abundance of amorphous-looking granules. A few granules around the periphery suggested a concentric lamellar structure (fig. 6). Inclusions of reticulocytes could be seen in some of the mast cells (fig. 7). Mastocytes containing a highly osmophilic structure in the cytoplasm surrounded by a membrane could also be observed occasionally (fig. 8)

Discussion

Systemic mastocytosis is an uncommon disease. Even in patients with clear clinical pictures we believe that its definite diagnosis is based on the identification of multifocal proliferation of atypical, immature or poorly

Fig 5 Mast cell. Granular polymorphism and microvilli transversally sectioned (asterisks). The arrow points to a granule of great size × 6,000. Bar equals 833 nm.

Fig 6. Mast cell with three granules (asterisks). The formation of peripheral loops (arrows) with the resulting circinate profile can be seen in one of them. × 40,000. Bar equals 125 nm.

5



6



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5



6



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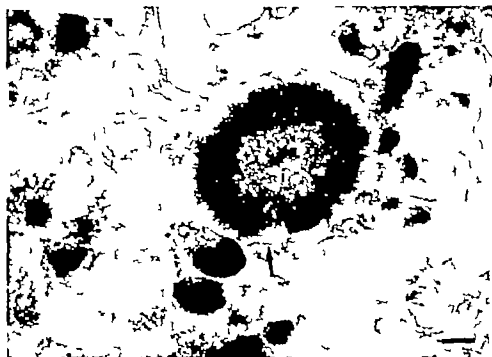


Fig 8 Mast cell Cytoplasmic fragment containing a highly osmiophilic inclusion of great size (I) surrounded by a membrane unit (arrow) × 15,000. Bar equals 333 nm.

developed mast cells. Proliferative mastocytosis should be distinguished from some basophilic conditions including basophilic tissular reactions

Cytological cytochemical and ultrastructural criteria confirmed the mastocytic nature of the cellular proliferation in our patient Cells were *highly positive to chloroacetate esterase stain* [22] *Furthermore the cel*lular ultrastructure revealed the special characteristics of the granules and the finger-shaped cytoplasmic projections in the periphery of the cells.

Waldenström s macroglobulinemia and other lymphoproliferative diseases [9 12] as well as aplastic anemia refractory anemia chronic hepatopathies, osteoporosis [6] etc are some examples of other clinical conditions besides mastocytosis that may be associated to a mast cell proliferation Macroglobulinemia could be disregarded because of the absence of monoclonal gammopathy in the patient s serum. The splenohepatomegaly excluded aplastic anemia The differential diagnosis was more difficult in the case of hepatic cirrhosis since splenohepatomegaly peripheral pancy

topenia and ethylic antecedents all pointed towards that condition. Unfor tunately a pathologic study of the liver could not be carried out because severe thrombocytopenia made liver biopsy unadvisable. Hepatopathy was considered improbable according to the lack of clinical symptoms of cirrhosis and the complete normality of the liver function tests.

In the above-mentioned clinical situations the mast cells in bone mar row do not usually comprise such a high percentage of the total. They are generally more mature in appearance than those found in our patient. The immaturity of the mastocytes and their presence in the peripheral blood are the key factors in defining the proliferative character of this disease. At the ultrastructural level the homogeneous content of the granules stands out, pointing to that they are probably immature cells since more developed ones offer a more varied internal structure [20] Only in a few granules a concentric lamellar structure can be observed.

There is little to be found in the literature on the ultrastructure of systemic mastocytosis. Some authors [13] have reported anything abnormal in the cytology of this condition. Others [18] however have discovered such anomalies as variegated morphology of the granules, paracrystalline structure similar to that of the eosinophils, and abnormally large microvilli intermingled with those of the neigboring cells [11] We frequently found erythrocytic inclusions within the mast cells. This observation was recorded only by MUTTER *et al* [10] in another case of systemic mast cell disease. Another submicroscopic morphological characteristic we did not find described elsewhere was a highly osmiophilic inclusion surrounded by a membrane unit, in some of the cells. We interpreted this finding as a *probable product of cellular secretion. Biochemical studies did not reveal* the mastocytic nature of the disease. They did not invalidate the diagnosis, however since normal values for these substances are frequently re corded even when a highly symptomatic clinical picture has developed [10] This may be true because rises in plasma concentrations are sporadic or more likely because the amines are released locally and in small doses not detectable by the analytical methods normally employed.

The accompanying hematological data consist of peripheral pancytopenia, bone marrow hypercellularity and marked overall dyshemopoietic features. This can be superimposed on refractory anemia and especially on chronic myelomonocytic leukemia in view of the existence of a splenomegaly and a significant peripheral mastocytosis [21] In our judgement, the proliferation of mast cells, in both its quantitative and qualitative aspects, relegates the refractory character of the anemic syndrome to a sec

ondary level which could be considered a paraneoplastic expression a pos sibility already suggested in the sideroblastic refractory type of anemia [5]

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Bone Marrow Morphology in Patients on Regular Haemodialysis Treatment

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Key Words. Anaemia of uraemia Bone marrow Eosinophilia Erythropoiesis Haemodialysis Megaloblasts Renal failure

Abstract Bone marrow smears from 57 patients with chronic renal failure were morphologically analysed. In 55 patients on regular haemodialysis there was a slight 'shift to the left and a significant eosinophilia within the granulopoiesis concurrently with slight megaloblastic changes of the erythroblasts. It is suggested that these abnormalities are caused by the dialysis procedure and not by the uraemia *per se*

The successive development of anaemia and renal osteodystrophy are regular clinical features in patients with chronic renal failure Bone marrow biopsy has been considered to be a reliable method of evaluating these patients and is a routine procedure in patients undergoing long term haemodialysis [8]

The aim of the present work was to investigate whether any characteristic changes in composition and morphology of the bone marrow will occur as results of the dialysis situation independent of the type of kidney disease.

Material and Methods

Patient 27 men and 30 women with chronic renal failure were included in this study Their age was 16–66 Hb was 35–140 (median 63) g/l The patient on regular dialysis (RDT) were dialysed twice weekly 9–11 h each time using the Gambro Lundia Nova 13.5 m dialyser They were allowed normal food except for excess sodium and potassium They were supplemented with 5 mg fol c acid nd one polyvitamin preparation containing 6 mg pyridoxin daily Iron was gi en intra e

nously when the iron saturation index (S-Fe/S-TIBC) was below 50%. The levels of folic acid in blood and of B in plasma were normal in all patients. Blood transfusions were only given when the patients suffered from pronounced fatigue or from angina pectoris. This was the case of 6 patients, and only once month or more infrequently

The patients were grouped as follows:

Non-dialyzed (nD). 6 men and 3 women aged 28–62 with various renal diseases before entering regular dialysis treatment. Hb was 60–117 (median 99) g/l. 7 patients were also included in the groups beneath.

Dialyzed (D). 25 men and 30 women aged 16–66 with RDT for at least 12 months during the years 1970–76.

Dialyzed with chronic interstitial nephritis (D-in). 2 men and 12 women aged 16–59 with Hb 44–87 (median 60) g/l.

Dialyzed with chronic glomerulonephritis (D-gln). 11 men and 4 women aged 22–61 with Hb 45-100 (median 73) g/l.

Dialyzed with polycystic kidneys (D-pck). 4 men and 3 women aged 55–66 with Hb 63–95 (median 68) g/l.

Dialyzed with chronic renal failure due to other conditions or with uncertain diagnoses (D-rf). 4 men and 8 women aged 20–56 with Hb 43-140 (median 55) g/l.

Dialyzed and nephrectomized (DN). 4 men and 3 women aged 20–55 with Hb 35–65 (median 50) g/l.

Dialyzed June 1976 (D_{76}). 5 men and 9 women aged 26–66 with Hb 43–74 (median 58) g/l. These patients are also included in the groups above.

Normals. 17 probands without perceivable haematologic disorders and with clinical diagnoses such as spondylolisthesis, cervical disc prolapse, adipositas, psychiatric disorders and unverified hypogonadism served as controls. Hb 121 166 g/l, WBC 2,200–8,300 and ESR 2 23 mm.

Bone marrow examination. 64 bone marrow smears were stained with May-Grünwald-Giemsa. A differential count of 1,000 nucleated cells was performed and the cells were classified according to HEILMEYER and BEGEMANN [10] and SJÖGREN [15]. By counting 1,000 erythroblasts the proportion of megaloblasts and the mitotic index were determined. Proerythroblasts and basophilic erythroblasts were pooled into one group designated basophilic erythroblasts. Evaluation of the percentage of sideroblasts was performed by counting 100 erythroblasts in bone marrow smears stained with 10% potassium ferrocyanide.

Statistics. Non-parametric statistics were used. The results are given as median (Q_2) and interquartile range. The Mann-Whitney two-tailed U test corrected for ties was used to assert significance of the results.

Results

There was a significant shift to the left within the granulopoietic part of the bone marrow of the patient group D_{76} compared to the normals and the *nD*. Thus the ratio between the precursor cells (myeloblasts + promye-

Table I Bone marrow data from 14 patients with regular dialysis in June 1976 and from 17 normals values give percentage of all nucleated cells and significance of difference between patients and normals

	D_{76} Q_1–Q_2–Q_3	Normals Q_1–Q_2–Q_3	
Myeloblasts	1.3–1 8–2.0	1 7–2.1–2.7	$p > 0.10$
Promyelocytes	3 7–4.3–5 0	2.1–3.0–3 4	$p < 0.002$
Myelocytes	8 7 10 6–12.2	8 7 9 8–13 6	$p > 0.10$
Metamyelocytes	7 0–8 1 10 0	9 6–10.6–1[illegible]9	$p < 0.01$
Band forms	14.5–17 4–20.5	14.4–15 8–17.[illegible]	$p > 0.10$
PMN neutrophils	9 4–14.[illegible] 17.3	11 6–14 4–19 6	$p > 0.10$
Eosinophils	3 6–4 4–7 3	[illegible]1 2.7–3 7	$p < 0.002$
Mast cells	*0.0–0 1–0.2*	*0.0–0.0–0.0*	$p > 0.10$
Erythroblasts	19 1– 1[illegible]– 4 6	18 0–20.5–[illegible]3 0	$p > 0.10$
Megaloblasts (% of all erythroblasts)	5 7 4–10.3	1 1 7–[illegible]3	$p < 0.00$[illegible]

Table II Frequency of eosinophils in bone marrow from 57 patients with chronic renal failure and from 17 normals values give percentage of all nucleated cells and significance of difference between patients and normals

Group	n	Q_1–Q_2–Q_3	
nD	9	2.6–3 4–4.6	$p > 0.10$
D-in	14	3.4–4 1–6.6	$p < 0.01$
D-gln	15	3.3–4.7–8.7	$p < 0.01$
D-pck	7	4 0–4.3–5.6	$p < 0.01$
D-rf	1	3 1 3 8–6.7	$p < 0.01$
DN	7	2.9–7 3–10.4	$p < 0.01$
Normals	17	2.1 2.7 3.7	

locytes + myelocytes) and the more mature cells (metamyelocytes + band forms) was 0 74 (Q_1–Q_3 0 72–0 88) in D_{76} compared to 0 66 (Q–Q_3 0.59–0 77) in the normals, $p < 0\ 01$ This difference seems to be caused by a raised frequency of promyelocytes in the patient group (table I)

There were significantly more eosinophils in the groups with regular dialysis compared to the normals and the *nD* (table I II) There were no significant differences within the *D*-group (table II)

Table III Frequency of megaloblasts and sideroblasts in bone marrow from 57 patients with chronic renal failure and from 17 normals values give percentage of all erythroblasts and significance of difference between patients and normals

Group	n	Megaloblasts Q_1–Q_2–Q_3		Sideroblasts Q_1–Q_2–Q_3	
nD	9	1 4–2.0–2.5	p > 0 10	25–39–50	p > 0.10
D-in	14	2.7 5 2–11 1	p < 0 002	63–73–83	p < 0.002
D-gln	15	3.0–5.2–6.9	p 0.002	66–75–77	p < 0.01
D-pck	7	4.9–5.3–11.8	p < 0 002	21 56–75	p > 0.10
D-rf	12	2 8–6 9–9.3	p < 0.002	63–83–86	p < 0.002
DN	7	4.3–6 9–7 9	p < 0 002	81–88–92	p < 0.002
Normals	17	1_ 1 7 2.3		28–42–60	

There was a significantly higher incidence of mast cells in the *D*-groups compared to the normals, $p < 0.005$ and also to the *nD* $p < 0.025$ (Chi-square test).

There were significantly more megaloblasts and sideroblasts in the *D*-groups compared to the normals and the *nD* (table III) There were no remarkable differences within the *D*-group. The megaloblastic tendencies was in concordance with MCV values between 93 and 114 fl (normal range 82–102).

There was no difference between the mitotic indices of the erythroblasts of the patients and the normals. Thus the median mitotic index was 2.9‰ (Q_1–Q 2.5–3.5) in the D_{rs} compared to 2.9‰ (Q_1–Q_3 _.8–3.3) in the normals.

The composition of the erythroblast series was apparently the same in the patients and the normals with, on average 31 4 / (Q_1–Q 29 1–34.5) and 30.5‰ (Q_1–Q_3 29.5–31 9) basophilic erythroblasts respectively The difference is not significant.

Discussion

Several mechanisms have been reported accounting for the pathogenesis of the pronounced anaemia in patients undergoing RDT The bone marrow of uraemic patients seems to contain erythroblasts with normal responsiveness to erythropoietin (EPO) but the levels of circulating EPO are low Besides serum factors inhibiting the EPO-stimulated erythro-

poiesis have also been found [7 16 17] An increased destruction of red blood cells is caused by the dialysis and by haemolysis initiated by the azotaemia [2 7] Sometimes there are laboratory signs of hypersplenism and in these cases splenectomy has given encouraging results [2] By supplementing the patients with folic acid soluble vitamins and iron other sources of anaemia such as blood loss in connection with the dialysis, blood sampling and bleeding could be corrected

The anaemia in patients on RDT has been described as normocytic and normochromic with no megaloblasts and normal percentages of side roblasts in the bone marrow [7 8] Evaluations of marrow iron stores have given evidence of iron overload however [7 8] This phenomenon is of course depending on the policy of iron supplementation and blood transfusions. Some authors have noticed varying degrees of megaloblastosis in the bone marrow of RDT patients [9 11] In some cases slight me galoblastic changes remained after folic acid therapy and it was postulat ed that these changes were caused by a uraemic toxin interfering with DNA synthesis [9] In the present investigation megaloblastic tendencies and slight megalocytosis were found concurrently with normal mitotic indices and normal frequencies of early erythroid precursors. The reason for these megaloblastoid changes is obscure and a tentative explanation may be the vitamin B_6 deficiency which is common in patients with urae mia [6] High percentages of sideroblasts in the patients on RDT are in agreement with findings of iron overload in the marrow caused by the liberal iron supplementation used at our unit

Within the granulopoiesis we found a slight but significant shift to the left During the dialysis there will be a loss of circulating granulocytes which adhere to or will be damaged by the dialysing surfaces [3 4 13] The transient neutropenia will then be compensated by an influx of neu trophils from the bone marrow [3 4] Our finding of a raised frequency of immature granulopoietic precursors are in agreement with high mitotic indices reported by others [3] A state of inflammatory response is also seen in the plasma protein patterns in these patients. Thus these observa tions may be signs of a stressed granulopoiesis.

Eosinophilia was a constant finding in this investigation and it has for merly been described also in a patient on chronic peritoneal dialysis [12] Quantitative observations on iliac bone marrow have given evidence of a raised frequency of mast cells in chronic renal failure with osteodystrophy [14] and this is verified in the present investigation It is known that eosi nophilia may be the result of a reaction of antigen and antibody of IgE

class fixed to the surface of mast cells [5] There are also observations that certain patients on heparin therapy appeared to exhibit an eosinophilia [1 12] A third explanation may be the finding of high frequencies of eosinophils adhered to the dialyser membranes thus stressing the bone marrow to produce more eosinophils [13]

This investigation gives evidence of certain abnormalities, probably reactive, which occur in the bone marrow in patients on RDT independent of the type of underlying kidney disease. We think these changes of the bone marrow morphology should be taken in account when performing a differential count. We intend to study the effect of vitamin B_6 supplementation on the megaloblastic changes observed.

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Acquired Aplastic Anaemia in Adults

II. Conventional Treatment: Retrospective Study in 40 Patients

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Key Words. Acquired aplastic anaemia in adults · Androgen treatment · Splenectomy

Abstract. The effect of conservative treatment of aplastic anaemia was evaluated retrospectively in 40 patients. No significant beneficial effect was provided by long-term high-dose oxymethalone in 20 patients or by metenolone, adrenosterolone, or testosterone in 14 patients. Splenectomy gave no improvement in the majority of cases, although in some it decreased the transfusion requirement. Immunosuppressive treatment was successful in 1 patient with positive LE phenomenon. Until specific treatment becomes available, the possibility offered by alternative treatment, e.g. bone marrow transplantation, in cases with poor prognostic parameters should be considered.

Introduction

The treatment of aplastic anaemia is a controversial subject, probably due to the heterogeneity of the pathogenetic mechanisms. Because of the rarity of the syndrome, adequately controlled clinical trials in a large number of patients are not feasible and retrospective studies are complicated by many evaluational problems. Several factors have affected the therapeutic results, e.g. the use of new antibiotics and of strict isolation techniques in combination with gastro-intestinal bacterial decontamination. Concomitantly important advances have been made in transfusion methodology supported by a rapidly expanding knowledge of the histocompatibility antigens [35].

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by testosterone propriate, after which the liver enzyme and bilirubin levels returned to normal.

Antibiotic treatment was selected on the basis of the results of the *in vitro* antimicrobial sensitivity tests; 'prophylactic' use was voided. White cell transfusions were given to treat resistant septicaemia in granulocytopenic patients. In most of the patients red cell transfusions were administered in the form of packed cells. For many transfusions, the material was freed of white cells by removal of the buffy coat, cotton wool (Leukopak®) filtration, or more recently with the filter described by DIEPENHORST *et al.* [9]. Platelet support was usually given with cells from random donors, but in several cases from HLA-matched sibs or non-related donors. Many patients were nursed in reversed isolation systems, either in the Isolation Ward [51] or 'isolation rooms' in the Department of Haematology or the Department of Infectious Diseases.

Results

The relevant clinical data concerning the results of the androgen treatment are shown in table I. The relatively high RI seen at the start in patient No 40 declined to very low levels during treatment. An improvement in the haematological status was defined as a rise of haemoglobin above 10 g°/₀, of the granulocytes above 1,500/mm³ and the platelets above 50,000/mm³. 13 patients received oxymethalone for at least 3 months, of whom 7 showed signs of haematopoietic recovery as indicated in table II. A rise in the haemoglobin level was invariably followed by normalization of the granulocyte counts, recovery of the platelet levels is less common. To investigate the effectiveness of androgen therapy we compared the occurrence of haematopoietic recovery in all of the patients who were still alive 3 months after diagnosis. This selection of course excludes the patients with an unfavourable prognosis, but includes all of those who could have benefited from androgen treatment lasting at least 3 months. Table III shows that in this group there was no significant effect of androgen treatment (regardless of the drug form). In an attempt to identify factors that might influence the 'response' to androgen treatment, we compared the patients' age, sex, aetiology and initial RI and granulocyte counts. No significant differences were found, although there proved to be more male patients in the 'responsive' group.

It seemed important to rule out differences in supplementary treatment (e.g. transfusions) that might have influenced the therapeutic results obtained in this disease during the last 10 years. Therefore, we compared the 3-month survival and the haematological recovery with and without androgens before and after January 1st 1971 (table IV). This date was

In addition to these developments, the use of high-dose androgen treatment was advocated by SHAHIDI *et al.* [39] SHAHIDI [41] and SANCHEZ MEDAL *et al* [36] More recently LI *et al.* [27] BOCK and HEIMPEL [4] GORDON SMITH *et al* [13] and WILLIAMS *et al.* [53] found no significant improvement in the prognosis of the patients treated with androgens. To evaluate the results of conventional treatment – i.e., all therapeutic measures except bone marrow transplantation – we reviewed the case histories of 40 patients suffering from aplastic anaemia treated in the Leiden University Hospital between January 1st 1964 and January 1st 1976

Patients and Material

All 40 patients suffered from aplastic anaemia as defined in detail elsewhere [18]. Briefly the diagnosis was based on (pan)cytopenia and marked cytologically and histologically demonstrated loss of haematopoietic parenchyme in bone marrow on more than one occasion, without signs or symptoms of deficiencies or of metabolic or malignant disease. Laboratory investigations included routine peripheral blood counts and May-Grünwald-Giemsa stained smears. Reticulocyte counts were performed after cresyl blue staining, corrected for haematocrit, and expressed as reticulocyte index [21]

$$(\text{RI} = \text{reticulocyte count } (^0/_{00}) \times \frac{\text{haematocrit}}{46\,(♂) \text{ or } 41\,(♀)}).$$

Normal value 10–40. As the most reliable criteria for the overall effective haematopoietic function we selected the RI and granulocyte counts. Statistical analysis was performed by χ^2 tests for group comparison (at one degree of freedom).

All patients received some form of treatment, e.g. transfusions and antibiotics, and most of them were also given androgens, often in combination with low-dose corticosteroids. This treatment included splenectomy in 7 cases. In 11 patients the prognosis was considered very bad and they were prepared for bone marrow transplantation. The selection for transplantation was dependent on the availability of a suitable donor and of adequate facilities in the hospital [17 43]

26 patients were given androgen treatment. Oxymethalone was used as first drug in 17 cases, metenolone in 4 nandrolone in 3, and methandrostenolone in 2 (table I). A second androgen treatment was given in 11 cases. In 5 patients the haematopoietic improvement obtained in the first treatment was sustained during the second irrespective of the drug form. In another 4 cases no improvement was observed during the first as well as the second treatment.

Patient No. 32 improved during a first treatment of oxymethalone but a subsequent relapse persisted despite a prolonged second course of the same drug. Elevation of the SGOT SGPT and bilirubin levels developed in some patients during oxymethalone treatment. In 4 patients this drug was replaced by metenolone and in 4

Table II. Haematological recovery after oxymethalone therapy

Patient No.	Hb (g %) before therapy	Hb > 10 g % after months	PMN[1] > 1,500/mm³ after months	Platelet > 100,000/mm³ after months	Total observation period months
15	8.3	6	6	24	> 59
18	4.8	3	3	9	> 69
20	6.8	9	( )		> 53
21	6.3	3	6	12	> 63
27	8.7	3	5		30
28	9.0	8	8	–	18
32	5.0	6	9		60
	Mean	5.4 ± 2.5	6.2 ± 2.1		
	Median	6	6		

No patient without erythropoietic recovery showed an increase in PMN.
From start of therapy PMN > 1,500/mm

Table III Influence of androgen therapy on haematological recovery

	Androgens total number patients	survived 3 months	haematol. recovery	No androgens total number patients	survived 3 months	haematol. recovery
	18	15	6	12	5	4
BMT	*8*	*5*	*3*	*2*	*2*	*0*
	26	20	9	14	7	4

BMT = Bone marrow transplantation haematological recovery = stable increase of Hb > 10 g/dl and PMNS > 1,500/µl.

chosen because since then the possibilities for collecting platelets from non-related HLA-matched donors have greatly increased. In addition, the application of gnotobiotic techniques led to much more effective isolation and decontamination procedures. The group since 1971 includes a remarkably high number of patients who survived beyond the first 3 months, although this difference is not significant. However the incidence of haematopoietic recovery after androgen treatment was not higher in this group. This is an indication that since 1971 more patients were adequately supported for the first 3 months after the diagnosis. These results

Table I Relevant data on 26 androgen-treated patients

Patient No.[1]	Age years[2]	Sex	Year[3]	Initial counts[4] Hb	RI	PMN	Androgens[5] first	second	Survival[6]	Other treatment[7]
1	43	m	1965	7.5	8	600	NA 3 −		4	
3	59	m	1966	[illegible]6	6	600	MA 3 −		3	
7	70	m	1969	6.0	17	[illegible],800	OX 2 −		[illegible]	
9	60	f	1973	9.2	0	300	OX ½ −		2	Sx (1½)
10	21	f	1974	8.7	27	1,500	OX 4 −		4	
13	26	m	1966	7 7	23	1,500	MA 6 −		34	Sx (6)
15	66	m	1966	8.3	9	1 000	NA 2 +	OX 59 +	121 +	
17	44	f	1968	7 1	2	1 800	OX 12 −		60	
18	50	f	1970	4 8	20	700	OX 69 +		73 +	
19	50	m	1970	7 1	23	1,300	OX 66 +		78 +	
20	6[illegible]	f	1970	6.8	8	1 800	OX 12 +	TP 41 +	75 +	
21	14	m	1971	6.3	[illegible]6	380	OX 63 +		67 +	
23	24	f	1971	5 8	26	1,200	OX 15 −		21	
24	29	f	1971	5 1	8	1,000	ME 15 −	OX 3 −	20	Sx (15)
25	68	m	1971	4 8	5	1 600	OX 9 −	TP 3 −	1	
27	61	m	1973	8.7	4	270	OX 7 +	ME 23 +	30	
28	64	m	1973	9.0	19	570	OX 9 +	ME 9 +	18	
29	47	m	1975	4.0	10	380	NA 4 +	ME 16 +	21 +	Sx (9)
32	16	m	1970	5.0	20	600	OX 4 +	OX 12 −	60	BMT (60)
33	26	m	1970	4 0	3	500	ME [illegible] −		3	BMT (3)
34	18	m	1971	6.9	2	90	ME 5 −	OX 2 −	7	BMT (5)
35	47	f	1972	9.9	1	400	OX ½ −		1	BMT (½)
36	14	f	1974	9.5	2	[illegible],400	OX 2 −	OX 10 +	28 +	BMT ([illegible])
38	23	m	1974	6.8	2	300	ME 8 −		4 +	BMT (5), Sx (6)
39	50	f	1974	8.5	2	200	OX 5 −		5	BMT ([illegible])
40	42	f	1975	8.1	72	1 800	OX 2 −	OX 4 −	19 +	BMT (2), Sx (4)

[1] Patient numbers are the same as in HAAK *et al* (1977) [18].

[2] Age at diagnosis.

[3] Year of diagnosis.

[4] Hb = g/dl RI = reticulocyte index = $\frac{\text{ret. count } (‰) \times \text{Ht PMN per mm}^3}{41 \text{ f or } 46 \text{ m}}$

[5] Androgen treatment OX = oxymetholone ME = metenolone NA = nandrolone MA = methandrostenolone TP = testosterone proprionate. Numbers refer to duration of treatment in months + = improvement − = *no improvement.*

[6] Survival in months after diagnosis + = alive.

[7] BMT = bone marrow transplantation Sx = splenectomy (in parentheses interval in months after diagnosis).

Table V The results of splenectomy (Sx) performed in 7 patients suffering from aplastic anaemia

Patient No.	Age years	Date diagnosis	Aetiology	Counts before Sx: Hb g%	R ‰	WBC μl	PMN μl	plat. μl	Sx (date)	Counts after Sx: Hb g%	R ‰	WBC μl	PMN μl	plat. μl	Last transf	Survival post Sx	Cause of death
9	60	3/73	ecl	5.7	0	1,350	410	7,500	17/04/73	10.5	0	2,500	500	27 000		2 m	bleeding
12	32	5/64	CAP	9.2	5	1,500	400	15,000	9/11/64	9.7	8	3 700	1,000	200,000	19/01/65	~11 y	
19	50	8/70	benzene?	10.8	20	9,000	?	22,000	1/09/70	9.7	13	3 100	500	27,000	1/06/71	5 y	improved after ox.
24	29	5/71	eci	7.5	4	2,100	700	15,000	1/10/72	8.5	5	2,600	500	47,000		2.5 m	bleeding
29	47	2/75	eci	10	0	2,500	200	10,000	1/09/65	10.8	0	200	20	12,000		18 d	viral pneumonia
38	23	12/74	ecl	8.1	60	990	470	40,000	31/05/75	7.9	40	3,000	1,000	9,000	7/12/75	>14 m	
40	42	3/75	ecl	5.5	0	880	30	50,000	30/06/75	6.2	20	3,300	800	20,000	1/06/76	>12 m	

Peripheral blood cell counts performed during the week before operation, usually after substitution. Platelet levels determined during *substitution therapy except patient No. 12 post-splenectomy*

Performed in peripheral blood at death or 3 months after the operation.

Table IV Haematological recovery and 3 months survival according to year of diagnosis

Year of diagnosis		Androgens			No androgens		
		total number patients	survived 3 months	haematol. recovery	total number patients	survived 3 months	haematol. recovery
1964–1970		9	7	5	7	3	2
	BMT	*2*	*0*	*0*	*2*	*1*	*0*
		11	7	5	9	4	2
1971–1975		9	8	1	4	2	2
	BMT	*6*	*5*	*3*	*1*	*1*	*0*
		15	13	4	5	3	2

do not support the hypothesis that long-term high-dose androgen treatment increases the incidence of haematopoietic recovery in patients suffering from aplastic anaemia.

Side Effects of the Androgen Treatment

In 6 of the 20 patients given oxymetholone SGOT and SGPT showed elevated levels after 1–9 months of treatment, and one of these developed a mild jaundice. In none of these cases could hepatitis-associated antigen be demonstrated. Screening for hepatic tumours was negative. The elevated levels subsided after discontinuation of the drug In 1 case extensive signs of pellosis hepatis were found at autopsy after 9 months on metenolone. Most of the treated patients showed signs of virilization and fluid retention 4 patients complained of severe muscle cramps during treatment with oxymethalone or metenolone. These complaints disappeared after lowering of the dose from 3 to 1–2 mg/kg.

Splenectomy

Table V shows the results of splenectomy which was performed in 7 patients. 4 of these 7 died, 3 of them within 3 months after the operation. There was no direct surgical mortality in this group The procedure was carried out after correction of the bleeding time with random or matched platelet transfusion. The blood counts in 1 case improved 2 months after the operation. 2 other patients showed a slow recovery which started a year after splenectomy during this period both were on androgens. 1 patient did not show significant improvement and required platelet support repeatedly

the patients treated with androgens. Data from small randomized clinical trials described by BOCK and HEIMPEL [4] and GORDON SMITH *et al.* [13] do not indicate significant improvement in the prognosis of aplastic anaemia under androgen treatment. HAST *et al.* [20] reported a multicentre study on the therapeutic use of high-dose androgen. The median survival was slightly longer than in other studies, but because there was no randomized control group, this result cannot be attributed to the androgen treatment alone. However in certain individuals the effectiveness of this treatment seemed indisputable [32] The cause of these discrepancies has not been elucidated. Experimental work indicates a complex mechanism underlying the action of androgens on haematopoiesis.

The 5-α-hydroxy derivates are thought to enhance erythropoietin production [41] and the 5-β-derivatives to trigger the stem cell compartment, probably directly [14 41] The influence of oxymethalone on urinary erythropoietin excretion in patients suffering from various forms of bone marrow insufficiency was studied by ALEXANIAN *et al.* [1] After 1 month of treatment 6 patients suffering from aplastic anaemia showed a marked increase in erythropoietin excretion, but no signs of haematopoietic recovery NEUMANN *et al.* [33] did not see increased serum levels of erythropoietin in 6 patients suffering from severe aplastic anaemia during androgen treatment. Combined studies of serum and urine levels are still awaited! The action of androgens on the stem cell compartment in human aplastic anaemia has not been studied in detail. MORLEY *et al.* [31] recently described a model of aplastic anaemia based on induction by busulphan in mice. A controlled trial of 17-nor-testosterone did not show a significant effect on the haematopoietic malfunction. GORSHEIN *et al.* [14] observed a stimulating effect upon erythropoietic responsive cells in mice. It is, however difficult to reconcile these short-term effects with the 3 months delay required to obtain a significant improvement in human aplastic anaemia [32] Our findings do not rule out the existence of some beneficiary influence of androgens on the course of some cases of aplastic anaemia. Hypothetically the stimulating influence on phagocytosis by mononuclear cells [12, 29] or the depletion of bone marrow lymphocytes [10] could alter the micro-environment and thus lead to more favourable conditions promoting the proliferation and differentiation of haematopoietic stem cells.

The side effects of androgen therapy include fluid retention and virilization. Both hepatic toxicity and tumour growth have been reported to occur during treatment with high dosages of 17 alkylated androgens [20

Miscellaneous Treatment

This included prednisone in various dosages. In several patients pyridoxine was administered during a 2-month trial but without response 1 patient showed a circulating anti nuclear factor and a positive LE test. She had been treated with carbamazepine (Tegretol®) for a post-traumatic epilepsy This drug has been reported to cause aplastic anaemia [28] and was also related to a relapse of systemic lupus erythematodes in 1 case [28] After discontinuation of the drug and the institution of long-term immune-suppressive treatment with prednisone and azathioprine, this patient recovered completely

Discussion

The pathogenesis of aplastic anaemia is a matter of speculation In many papers on the course and prognosis of this disorder it is suggested that the mechanism is probably heterogeneous [18 27 42, 53] The prompt haematopoietic recovery after a bone marrow graft from a monozygous twin has been put forward as evidence for a failure of the multi potential stem cell [34 46] On the other hand, the failure of such a graft led TRENTIN [48] to perform extensive studies on micro-environmental influences on stem cell proliferation and differentiation.

Damage to the vascular component of the micro-environment [24] or an inhibitory action of lymphoid cells [2, 19] present in this environment, have been thought to depress normal haematopoiesis. The evidence for an immunologic auto-destructive mechanism in some cases is corroborated by the recovery of the autologous bone marrow after very intensive chemotherapy [45 47] or ATG pre-treatment [22] before bone marrow transplantation Besides these possible mechanisms, humoral disregulations have been proposed but never demonstrated [42] At present, no scientific basis for specific treatment is available The use of high-dose androgen therapy was advocated by SHAHIDI and DIAMOND [40] and SANCHEZ MEDAL *et al* [36] The latter authors reported a response of 50% to several androgens [36 37] Close scrutiny of these data, as carried out by STOHLMAN [42] shows that this result is not obtained in patients with an unfavourable prognosis based on low peripheral blood counts. This is in agreement with the findings of NAJEAN *et al* [32] and DAVIS and RUBIN [7] WILLIAMS *et al* [53] recently described a non randomized study in a large group of patients, the results showing an even worse prognosis in

Treatment with high dosages of corticosteroids and azathioprine was associated with a marked improvement in 1 case in which clear-cut evidence of auto-immunity was obtained. In other patients corticosteroid therapy in either high or low dosages was not followed by dramatic improvement. This is in agreement with results of other authors [15, 26, 27 50]. The addition of corticosteroids to androgens only aggravates the virilizing effects according to GARDNER [11] who suggested that steroid therapy is only indicated in case of marked haemolysis.

We conclude from our data that neither androgen therapy nor splenectomy seems to contribute to better chances of survival in aplastic anaemia in general. The syndrome of aplastic anaemia probably has a heterogeneous pathogenesis for which no specific forms of conservative treatment are available. In view of the many side effects of long-term high-dose androgen therapy and its equivocal therapeutic value, as observed in retrospective and prospective studies besides our own, we urge that its liberal use be reconsidered. Our data also suggest that although improvement in supportive care may decrease the early mortality after diagnosis, the incidence of haematopoietic recovery is not increased. Recently we described prognostic parameters which may improve the selection of patients with a poor prognosis [18, 49]. In this group more aggressive treatment, i.e. bone marrow transplantation, seems warranted [6].

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23 37] We found elevated SGOT and SGPT levels in about 30% of our patients treated with oxymethalone. These changes were reversible after discontinuation of the drug Hepatoma was not observed KÜHBOCK *et al.* [25] and BAGHERI and BOYER [3] suggested that a severe toxic destruction of liver parenchyme might be followed by peliosis hepatis. We found evidence of this complication at autopsy in 1 patient. Apart from the report by DELAMORE and GEARY [8] no data suggesting a 'leukemia-inducing activity' *of androgens in classic aplastic anaemia has been described.* The results of our retrospective non randomized study do not contradict the findings of WILLIAMS *et al* [53] BOCK and HEIMPEL [4] and GORDON SMITH *et al* [13] in failing to show a significant effect on patient survival and stable haematopoietic recovery of high-dose androgen therapy The side effects of this treatment may contribute to the morbidity In particular by the virilizing action in women. Both hepatoma and *peliosis hepatis* are rare but serious complications of this treatment.

The increased survival rate despite a lower incidence of haematological recovery in the early period after diagnosis (table IV) may partially be due to more effective transfusion techniques in recent years. The almost complete removal of leucocytes from packed red cell concentrates, now obtained with cotton wool filters postpones the development of allo-antibodies [9] The preparation of leucocyte-poor platelet transfusions appears to contribute the prevention of sensitization against HLA antigens. The development of anti-HLA antibodies is associated with a shortened platelet survival [30] It has been shown that platelet support from HLA matched unrelated donors will maintain adequate thrombocyte levels in heavily sensitized patients [30 44 55] Another factor that may have contributed to the decreased early mortality is the development of better isolation and decontamination techniques. The risk of bacterial infection is inversely correlated with the number of peripheral granulocytes [5] The application of laminar flow isolators and various forms of antibiotic decontamination has reduced the incidence of major bacterial and fungal infection [16 38] *It has been shown that manifest infection can also increase* the incidence of severe haemorrhage [54]

Splenectomy led to definite improvement in 1 patient out of 7 in our series The absence of a direct influence on the haematological status in this case confirms the findings of VINCENT and DE CRUCHY [50] GROSS *et al* [15] and VAN DER WEIJDEN and FIRKIN [52] On the other hand splenectomy *does* improve *the efficiency of transfusions. The platelet recovery* after transfusion in particular is increased in these patients.

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Fletcher Factor Deficiency – Detection of a Severe Case in a Population Survey

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Key Words. Fletcher factor · Haemarthrosis and haematoma · Factor XI · Factor XII

Abstract. The first case of Fletcher factor deficiency from the African continent is described. This was the only case of symptomatic Fletcher factor deficiency detected in a total population survey of 40,522 persons. This patient differs from other reported cases in that the child had symptoms of severe bleeding defect such as recurrent haemarthrosis and haematoma. The clinical features appear to improve with age. Both the PTT 'time course' and cold-induced EACA acceleration of the thromboplastin time are useful diagnostic tests for detecting homozygous patients. Our results confirm an earlier report that the EACA test is a sensitive test for detecting heterozygotes.

Fletcher factor (prekallikrein) deficiency is generally thought to be asymptomatic [5, 6] and cases reported so far have usually been detected by chance. This communication, which is, to our knowledge, the first case report of Fletcher factor deficiency from the African continent, describes a patient who presented with severe symptoms namely recurrent haemarthrosis and haematoma. He was detected in the course of a total population survey for the prevalence of haemostatic disorders in a defined community [3]. Results of family studies are also given.

Materials and Methods

Population Survey

The method of survey which was devised is described in full elsewhere [3]. The object was to determine the prevalence of haemophilia in a clearly defined Nigerian

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Table I. Results of screening tests on KO and immediate relations

	PT ratio	PTT (K) + mean difference	and 50 50	1/20 VIII difference	1/20 IX difference	Thrombin time sec	P & P test % activity
Control		33.5	33.5	41.1	40.3	10	90
Patient	1.09	+ 3.9	0	– 5.7	+ 1.5	9.1	80
Mother	1.01	– 1.9		+ 4.0	+ 3.0	8.0	90
Father	1.02	+ 0.6			–	8.1	75
Uncle	1.02	+ 9.0					–[a]
Normal							

50 50 = mixture of equal parts (v/v) of patient and control plasma. Test was performed only on control and patient samples. PT & PTT (K) = see text. P & P test = prothrombin and proconvertin test. 1/20 VIII = a dilution of 1 part of test in 20 parts of substrate plasma. This is a useful and sensitive screening test of specific factor deficiencies. The result of this and orthodox PTT is expressed as difference between the mean clotting time of patient and of control plasma samples [4].

Mean PTT (K) 33.6 – 3.6 [3].

Test were considered unnecessary

Results

Pre-transfusion haematological parameters were as follows: haematocrit (PCV) 29% there was neutrophil leucocytosis with a total white cell count of 15,570/μl, and blood film showed evidence of increased platelets although platelet count was, for technical reasons, not performed on the sample. His haemoglobin electrophoresis was homozygous A. His PT ratio was 1.0 and PTT difference was + 3.0 sec (normal > 10 sec [3]). Screening tests for factors XII, XI and X deficiency using appropriate substrate plasma gave normal results.

Results of tests of haemostasis are summarised in table I. The partial thromboplastin test 'time course' procedure shows clearly the progressive shortening of the PTT (K) clotting time with incubation (fig. 1). At 1 min incubation, the difference between the patient and control PTT (K) was 40 sec; it was 35 sec at 3 min incubation but dropped to 7.5 sec at 6 min and 2.75 sec at 10 min. The difference values between the father's clotting times and those of the control at the same incubation times were respectively 7.5 3, 1 75 and 0.75 sec. The difference between the control

community so as to obtain an estimate of the prevalence of this disorder in the population as a whole.

The survey was based on obtaining a history of prolonged post-circumcisional bleeding or death from such bleeding, from all parents in some villages in Ibarapa district in Western Nigeria. These were defined as 'positives' in the survey. They were requestioned closely by one of us (E. M. E.) and blood samples were then collected by clean venepuncture from those who appeared to have unequivocal history of prolonged bleeding. Plasma obtained from such samples (1 part of 3.8% citrate to 9 parts of blood) were tested by the prothrombin time (PT) and partial thromboplastin time (PTT) as initial screening tests for evidence of haemostatic failure. This survey method was found to be useful in the environment where medical facilities were extremely limited. It succeeded in raising the level of awareness in the population to the symptoms of haemostatic failure.

Patient

K. O., a male Nigerian child aged 5 months, was first seen in Lanlate Health Centre which was located in our survey district. The mother had taken him there during the survey period when she noticed that he had been bleeding from an injection site for 2 days. The injection was ordered for initial management of 'cellulitis' of the entire right hand. There was no history of trauma to the hand. The child was then sent to the University College Hospital (UCH), Ibadan, for investigation of the bleeding defect. Since we found no evidence of continuing bleeding, screening tests of haemostasis gave normal results, and the haematocrit of 32% was adjudged to be reasonably satisfactory, the mother was reassured and the child was sent home to be followed up at the Health Centre. 2 weeks later, he was brought back with marked painful swelling of the right elbow. There was, again, no history of trauma. Clinically, there was shiny swelling of the right elbow and gross painful swelling (haematoma?) of the right thigh. There was mareld pallor of the mucus membranes. It was noticed again that the child had still not been circumcised, contrary to the usual custom in his area of birth where most male children were circumcised by the age of 2 months unless there were special reasons for delay such as prolonged bleeding or bleeding to death of an earlier male child [3].

Haematology and Haemostasis Tests

Blood samples were collected by clean venepuncture from the patient, his parents and from an immediate paternal uncle – the father's elder brother. Each sample was mixed with ethylenediaminetetraacetic acid (1 mg/ml) for routine haematology, and 3.8% sodium citrate (1 part of citrate to 9 parts of blood) for coagulation studies. Routine haematological parameters were obtained by standard procedure [2]. Tests for haemostatic defect, including platelet count, PT, orthodox PTT with kaolin – PTT (K) (i.e. after preincubating the plasma with kaolin for 9 min), thrombin time, screening tests for factors VIII, IX, X, XI and XII, deficiency prothrombin and proconvertin test, were done [4]. Presumptive tests for Fletcher factor deficiency such as PTT (K) at shorter incubation time [1, 6] modified by completing the clotting time of fractions after different incubation periods and therefore obtaining a 'time course' was done. The 'cold-induced EACA acceleration' test [7] was also done.

Table I. Results of screening tests on KO and immediate relations

	PT ratio	PTT (K) + mean[1] and 50-50 difference		1/20 VIII difference	1/20 IX difference	Thrombin time sec	P & P test % activity
Control		33.5	33.5	41 1	40.3	10	90
Patient	1.09	+ 3.9	0	− 5.7	+ 1.5	9.1	80
Mother	1.01	− 1.9		+ 4 0	+ 3.0	8.0	90
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Uncle	1.02	+ 9.0					
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Test were considered unnecessary

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Results of tests of haemostasis are summarised in table I. The partial thromboplastin test 'time course' procedure shows clearly the progressive shortening of the PTT (K) clotting time with incubation (fig. 1). At 1 min incubation, the difference between the patient and control PTT (K) was 40 sec. It was 35 sec at 3 min incubation but dropped to 7.5 sec at 6 min and 2.75 sec at 10 min. The difference values between the father's clotting times and those of the control at the same incubation times were respectively 7.5 3 1 75 and 0.75 sec. The difference between the control

Table II Effect of different concentrations of EACA and temperature on the PTT clotting time (sec) of Fletcher factor deficient plasma and control

Epsilkapron (EACA)	KO	Mother	Father	Uncle	Normal
5 mg/ml					
Immediate	45.2	35 0	30.0	34.3	38.8
22 h (4°C)	44.5	36.2	32.8	*21.8*	*26.2*
22 h RT	68.2	41 4	39 0	30.8	38.2
10 mg/ml					
Immediate	38.5	34.2	28 4	28.2	39.5
22 h +4 C	44 5	36.2	25 0	*20.6*	*25 4*
22 h RT	55 4	36.8	39 0	31 4	32.8
20 mg/ml					
Immediate	46.4	29.2	33.6	33.8	42.0
22 h +4°C	32.4	30.2	*24.8*	*21.2*	25.4
22 h RT	51 0	41.2	38.5	31 0	34 6
40 mg/ml					
Immediate	42.6	26.2	–	34.3	41 4
22 h +4°C	36.4	30.0	–	*4.0*	*25.0*
22 h RT	43.2	37 9	–	32.2	37 0

Note Uncle was the father s brother EACA = Epsilon amino caproic acid.

and mother's results were not as clear-cut. Table II summarises results of cold-induced EACA acceleration test. With EACA at a final concentra tion of 5 and 10 mg both parents appear to have heterozygotes for Fletcher factor deficiency

Discussion

Previous reports of Fletcher factor deficiency patients had suggested that the deficiency was usually asymptomatic [1 6] In this report, the pa tient repeatedly presented clinically with bleeding problems characterised by recurrent haemarthrosis and haematoma. The lesions subsequently re-solved completely He did not, however bleed excessively when he was circumcised under close supervision in hospital. He is now 4 years old but has had no recurrence of severe symptoms although laboratory evidence

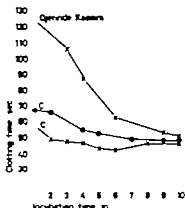


Fig. 1 PTT test with kaolin on citrated plasma samples of controls and the patient. *The difference between the patient and control clotting times is more clear-cut* after 6 min preincubation at 37 °C.

of the defect remains. His clinical course suggests improvement with age. It is not clear why he had such severe symptoms initially Modified PTT test and cold-induced EACA acceleration of the thromboplastin time procedures were employed in this study Both methods were found to be sensitive in detecting the homozygous case. If factor XII or XI deficiency are excluded by use of appropriate tests, the PTT 'time course could be a useful, simple and sensitive test for detecting Fletcher factor deficiency Our results also confirm the original suggestion of STORMORKEN and ABILDGAARD [7] that the 'EACA test is a sensitive procedure for detecting heterozygotes (table II).

A total of 40,522 persons were covered in the total population survey for the prevalence of haemophilia presenting as prolonged post-traumatic bleeding in Igbo-Ora area of Nigeria from which this single case of severe Fletcher factor deficiency was detected.

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Varia

2nd International Symposium on Therapy of Acute Leukemias

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The symposium is organized by the Institute of Hematology of the University of Rome with international participation. It will include Plenary Sessions, Workshops and Informal Sessions, covering all aspects of treatment of acute leukemias. For detailed program and further information apply to Prof. *Franco Mandelli*, Organizing Committee, Cattedra di Ematologia, Via Lancisi 3, *00161 Rome* (Italy).

Announcement

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For further information regarding membership, registration and submission of abstracts, contact the organizing committee of the Second Meeting of the Mediterranean Blood Club, PO Box 983, *Jerusalem* Israel.

8th Congress of the Hungarian Haematological Society
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Main topics: aplastic anaemias, cell membrane. Symposia: Application of isotopes in haematological diagnosis; Role of cytochemistry in the differential diagnosis of haematological diseases; Haematological cytogenetics. Language: Official language will be Hungarian, but papers may also be read in English and Russian.

For further information, please contact the secretary of the Congress, L. Tarján-Nagy, 3rd Department of Medicine, Semmelweis University Medical School, Mező Imre út 17 *H-1081 Budapest* Hungary

Erratum

In the article entitled 'Inherited Erythrocyte Pyruvate Kinase Deficiency Studies on 15 Members of Two Related Families' by M. Dacal, F. Carestiari, M. Bosati, P. L. Rossi Ferrini and G. Forconi, published in Vol. *57* 37–46 (1977), there are two errors:

page 40, table II, and page 41, line 13, GSSG-R instead of 6PGD·
page 42, footnote 1 to table III, 90 sec instead of 90 min.

Index rerum ad Vol. 58

Bearbeitet von G. Bonna, Basel

(B) = Book reviews Buchbesprechungen Livres nouveaux

Index autorum ad Vol. 58